METABOLIC INTERRELATIONS

WITH SPECIAL REFERENCE TO

CALCIUM

Transactions of the Fourth Conference

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ERRATA

FOR THE TRANSACTIONS OF THE THIRD CONFERENCE ON METABOLIC INTERRELATIONS, NEW YORK, N Y, JANUARY 8 9, 1951

1 The Editor regrets that *the name of Dr Harold C Hodge was incorrectly spelled* in the Table of Contents (page 2), in the title of the presentation (page 190), and in the running headings of this presentation (pages 193, 195 197, 199 201, and 203) The name was correctly spelled in the list of Participants and elsewhere in the discussions

2 *The address of Dr Frederic C Bartter was incorrectly given* in the list of Participants The correct address is U S Public Health Service Hospital, Wyman Park Drive and 31st Street, Baltimore 11 Md

JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT SMITH
Medical Director

As an introduction to these Transactions of the Fourth Conference on Metabolic Interrelations I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program. We are interested, first of all, in furthering knowledge about metabolic interrelations and to this end the participants were brought together to exchange ideas, experiences, data and methods. In addition to this particular goal, however, there is a further and perhaps more fundamental aim which is shared by all our conference groups. This is the promotion of meaningful communication between scientific disciplines.

The problem of communication between disciplines we feel to be a very real and a very urgent one, the most effective advancement of the whole of science being to a large extent dependent upon it. Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another, channels must be established for the most relevant dissemination of this knowledge.

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress. Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough. For example, today medicine must be well versed in nuclear physics because of the tracer technics and the injury which can result from radiation. At the other extreme, medicine is certainly a social science and, through mental health, must be concerned with economic and social questions. The answer, then, is not further fragmentation into increasingly isolated specialties, disciplines, and departments, but the integration of science and scientific knowledge for the enrichment of all branches. This integration, we feel, can be encouraged by providing opportunities for a multi-professional approach to given topics.

Although the fertility of the multi-discipline approach is recognized, adequate provision is not made for it by our universities, scientific societies, and journals. And perhaps the presence of other hindering factors must be admitted. Partly semantic in nature, they may also to some degree be psychological. Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar. By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus our meetings are in contrast to the usual scientific gatherings They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice For this reason we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will come prepared not to defend a single point of view but to take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories

We have now thirteen groups functioning under the Conference Program on the following topics adrenal cortex aging blood clotting cold injury connective tissues consciousness cybernetics infancy and childhood liver injury metabolic interrelations nerve impulse renal function and shock and circulatory homeostasis When a new conference is organized the Chairman in consultation with the Foundation selects fifteen scientists to be the nucleus of the group and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technic A limited number of guests are invited to attend each meeting but for the purpose of promoting full participation by all members and guests attendance at any meeting is limited to twenty five It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

The transactions of these meetings are recorded and published This is done because the Foundation wishes to make current thinking in a field available to all those working in it and because it believes that conveying to those in other fields who are concerned with science for example government officials administrators and so forth the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science but equally essential is the intuitive or creative aspect Research is as creative as the painting of a portrait or the composing of a symphony Although logic is of course necessary in order to rearrange to test and to validate research thrives on creativity which has its source in unconscious nonrational processes Unfortunately however in the finished products which are presented to the world through research reports this integral part of scientific endeavor is shriveled by the cold white light of logic By preserving the informality of our conferences in the published transactions we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

CARTILAGE AND BONE MATRIX CHEMICAL STRUCTURE, FORMATION, AND DESTRUCTION[1]

RICHARD H FOLLIS, JR

From the Department of Pathology The Johns Hopkins University School of Medicine and the Johns Hopkins Hospital Baltimore Maryland

Armstrong Dr Follis will you begin?

Follis When Dr Armstrong asked me to lead off this discussion on cartilage and bone matrix its chemical structure formation and destruction it did not take me long to realize how audacious I had been in accepting But in placing my head on the block if I can arouse discussion and also set a foundation for the conference today I will feel I have accomplished the purpose Dr Armstrong had in mind

Before proceeding I think we have to define cartilage and bone *matrix* I look on it as a material between the cartilage cells and the bone cells which is made up of fibers and interfibrillary material In our presentation we are going to take up first the micro and molecular structure of these materials and then go on to discuss what little is known of the formation and destruction of them This is putting the cart a little before the horse but I think it is the best way to proceed

The Structure of Cartilage Matrix

When one examines a paraffin section of cartilage say that from a rib with the light microscope one sees cells which vary in size from those which are very small and undifferentiated to those which are very large (the so-called hypertrophic cells) and lined up in rows at the cartilage shaft junction Between all of the cells there is what appears to be a homogeneous material which is bluish staining with hematoxylin Closer inspection however particularly in certain areas such as those between the rows of hypertrophic cells reveals faintly staining fibers (Figure 1) Such fibers and others elsewhere may be more clearly brought out by specialized technics such as enzymatic or alkali digestion followed by silver impregnation They also become more prominent in polarized light If certain stains such as toluidine blue are applied to ordinary formalin fixed

[1]Certain of the investigations reported herein were supported by grants from Mead Johnson and Company and the Florida Citrus Commission.



Fig 1 Cartilage Treated to Bring Out the Structure of the Matrix

A Cartilage stained with hematoxylin and eosin to bring out the definite though indistinct fibrillar structure of the matrix *B* Cartilage which has been digested with pancreatin and then stained with silver to bring out the fibrillar pattern more distinctly than in *A*

paraffin sections the matrix that is the fibers and interfibrillary material is found to be metachromatic diffusely so save that one can still see the faint outlines of fibers However if one examines a frozen section of unfixed cartilage one finds an important difference the matrix between the cells fails to take the dye as readily as do the capsular portions of the cells This is particularly striking in the area of hypertrophic cells and was commented upon by Dr Rubin[2] at this meeting two years ago, at which time its possible bearing on the calcification mechanism was conjectured *We shall have more to say about the phenomenon of metachromasia* a little later

If one now passes from the light to the electron microscope, one can with appropriate preparations gain further information concerning the fibers Those which have been isolated from cartilage and photographed by Wolpers[3] conform with the structures isolated from skin collagen which

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[2]Rubin, P S and Howard J E Histochemical Studies on the Role of Acid Muco-Polysaccharides in Calcifiability and Calcification *Trans Macy Conference on Metabolic Interrelations* 2 155 166 (1950)

[3]Wolpers C The Cross Striation of the Connective Tissue Fibril *Virchows Arch f path Anat* 312 292 (1944)

have been so intensively studied by Schmitt Gross and their associates[4 6] Fibers from skin collagen have a banded appearance with a periodicity in the range of 640 Å Each period consists of a light and a dark band If the preparations are suitably stained a more detailed intraperiod structure may be revealed, for instance six intraperiod bands have been observed

Hastings This is skin collagen?

Follis Yes skin collagen

Prior to the utilization of the electron microscope a good deal of information concerning the molecular structure of skin collagen had been obtained by the use of x ray diffraction Just before Schmitt *et al*[4] had identified 640 Å periodicity in collagen fibrils Bear[7] had noted this sequence in x ray diffraction images We shall not go into the complexities of this technic or its interpretation It is sufficient to summarize that by means of wide angle x ray diffraction one can interpret the fibers observed in the electron microscope as aggregates of polypeptide chains each having a thickness of 10 to 11 Å and being not over 800 Å in length We shall come back to this problem again shortly

Current evidence[8] based on chemical analysis appears to indicate that there are two principal components of cartilage matrix a protein (collagen) and a polysaccharide (chondroitin sulfuric acid) Certain amounts of lipoid and minerals are also present but these need not concern us here

Because of their similar amino acid content the collagens derived from skin bone cartilage and so forth would appear to be very closely related if not identical It is because of this that we feel one is justified in translating observations made on skin collagen to cartilage and bone Collagen is a unique protein because of its amino acid pattern[9] In the first place it has an extremely high glycine content (approximately 25 per cent) Secondly it contains large amounts (approximately 30 per cent) of the two

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[4]Schmitt F O Hall C E and Jakus M A Electron Microscope Investigations of the Structure of Collagen *J Cell & Comp Physiol* 20 11 33 (1942)

[5]Schmitt F O and Gross J Further Progress in the Electron Microscopy of Collagen *J Am Leather Chemists Assoc* 43 658 675 (1948)

[6]Gross J and Schmitt F O The Structure of Human Skin Collagen as Studied with the Electron Microscope *J Exper Med* 88 555 568 (1947)

[7]a Bear R S Long X Ray Diffraction Spacings of Collagen *J Am Chem Soc* 64 727 (1942)

b Bear R S X Ray Diffraction Studies on Protein Fibers I The Large Fiber Axis Period of Collagen *J Am Chem Soc* 66 1297 1305 (1944)

[8]Partridge S M The Chemistry of Connective Tissues I The State of Combination of Chondroitin Sulphate in Cartilage *Biochem J* 43 387 397 (1948)

[9]Neuman R E The Amino Acid Composition of Gelatins Collagens and Elastins from Different Sources *Arch Biochem* 24 289 298 (1949)

imino acids, proline and hydroxyproline[10] Hydroxylysine is said to be found only in collagen[11] In addition, it lacks cystine and tryptophane and contains only small amounts of tyrosine and methionine[9]

A good deal of attention has been given to the possible configuration of the amino acids in the polypeptide chains which make up collagen Because of certain regularities in the distribution of amino acids in collagen (gelatin), Bergmann[12] formulated what has come to be known as the 'periodicity hypothesis and suggested possible repetitive arrangements of amino acids in the polypeptide chain It is doubtful however, that any uniform periodicity prevails The presence of the imino acids proline and hydroxyproline does change the spacings along the fiber axis In an extended peptide chain, the length of one amino acid residue is 3 4 A X ray analyses of collagen show a shorter spacing of 2 86 A which is thought to be due to the presence of so many imino acid residues[13] (Figure 2) To conform to this Pauling[14] has recently formulated a structure for the collagen fibril which he postulates is comprised of three polypeptide chains bound together by hydrogen bonds each of the three polypeptide chains being coiled into a helix so that a cylindrical molecule is formed (Figure 3) These molecules are arranged in hexagonal packing to form a fiber of tendon or connective tissue

In addition to the hydrogen bonds referred to above, there are doubtless salt linkages between polar side chains It is the configuration or pattern of the amino acid residues which gives the x ray diffraction image and the intraperiod bands in the electron microphotographs Additional study may elucidate this fine structure further since the side chains may be modified by certain chemical procedures[15]

The other important component of cartilage is chondroitin sulfuric acid a long chain polysaccharide composed of residues of glucuronic acid and acetyl-galactose amine sulfuric acid which according to Dr Kurt H

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[10]Bowes, J H and Kenten R H The Amino acid Composition and Titration Curve of Collagen *Biochem J* 43 358 365 (1948)

[11]Rees M W The Estimation of Threonine and Serine in Proteins *Biochem J* 40 632 640 (1946)

[12]Bergmann M Complex Salts of Amino Acids and Peptides II Determination of *l* Proline with the Aid of Rhodanilic Acid The Structure of Gelatin *J Biol Chem* 110 471-479 (1935)

[13]Astbury, W T Croonian lecture On the Structure of Biological Fibers and the Problem of Muscle *Proc Roy Soc London Series B* 134 303 328 (1947)

[14]Pauling L, and Corey R B The Structure of Fibrous Proteins of the Collagen Gelatin Group *Proc Nat Acad Sc* 37 272 281 (1951)

[15]Bear, R S X Ray Diffraction Studies A Review of Recent Researches which Concern Collagen *J Am Leather Chemists Assoc* 46 438-445 (1951)

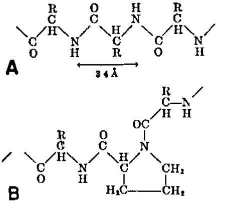


Fig 2 The Effect of Imino Acids on the Fiber Axis Spacing of a Polypeptide Chain

A Polypeptide chain composed of amino acids in which the fiber axis spacing is 3 4 Å *B* Proline containing peptide chain in which the fiber axis spacing is reduced to 2 86 Å by the presence of imino acids

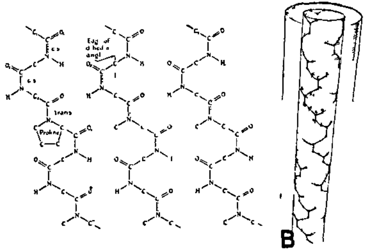


Fig 3 The Structure of a Collagen Fibril Proposed by Pauling[34]

A Pauling s representation of the configuration of polypeptide chains held together by hydrogen bonds in the collagen gelatin three chain helix *B* Diagrammatic representation of the collagen three chain helix according to Pauling [Reproduced by permission from Pauling L and Corey R B The Structure of Fibrous Proteins of the Collagen Gelatin Group *Proc Nat Acad Sci* **37** 272 (1951)]

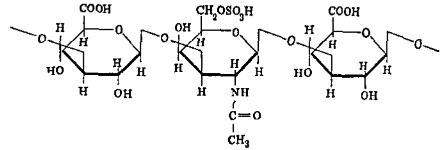


Fig 4 The Structure of Chondroitin Sulfuric Acid Proposed by Kurt H Meyer[16]

The long polymerized chains are composed of alternating molecules of glucuronic acid and acetyl galactose amine sulfuric acid [Reproduced by permission from Meyer K H Odier M and Siegrist A E The Constitution of Chondroitin Sulfuric Acid *Helv chim acta* 31 1400 (1948)]

Meyer[16] of Geneva has the configuration shown in Figure 4

Inasmuch as Dr Karl Meyer is going to discuss this polysaccharide at length I shall not go into the important subject as to the possible combination of chondroitin sulfuric acid and collagen However, mention should be made of certain very important recent observations by Schmitt, *et al*[17] In brief it has been found that if the corium is extracted with citrate buffer (pH 3 0 to 4 5) the extract after dialysis against water yields material which on electron microscope examination reveals two types of fibrils those having a period like collagen (640 Å) and a second and new type averaging 2000 3000 Å in periodicity This they call the 'long spacing' or LS fibril It was found further that if mucoprotein from plasma or skin were added to an acetic acid filtrate of rat tail tendon (rat tail tendon dissolved in acetic acid) a heavy fibrous precipitate appeared after dialysis against water This precipitate consisted almost entirely of LS fibrils Such observations suggest that mucoprotein or a component of mucoprotein may be concerned with the formation of collagen fibrils

Now cartilage has one property which is common to materials which contain mucopolysaccharides—it is not unique in that respect—and that is *the property of being metachromatic Thus if you stain cartilage after* fixation with certain dyes (the one usually used is toluidine blue), certain components of the tissue stain blue, but the cartilage takes a uniform red

[16]Meyer, K H Odier, M E and Siegrist A E The Constitution of Chondroitin Sulfuric Acid *Helv Chim Acta* 31 1400 1419 (1948)

[17]Highberger, J H Gross J and Schmitt F O The Interaction of Mucoprotein with Soluble Collagen an Electron Microscope Study *Proc Nat Acad Sc* 37 286 291 (1951)

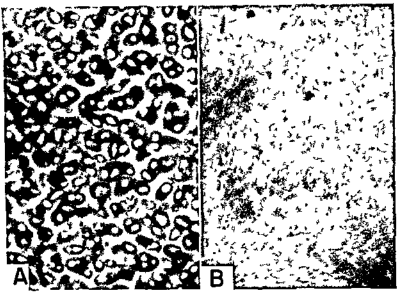


Fig 5 The Effect of Enzymatic Digestion on the Localization of Metachromasia in Cartilage

A Unfixed frozen section of costal cartilage stained with toluidine blue Note the aggregation of metachromatic staining material about the cartilage cells *B* Section similar to *A* incubated with trypsin The metachromasia has decreased and the pericellular aggregation seen in *A* has disappeared

stain If you take fresh material, that is a frozen section of unfixed cartilage, and stain it with toluidine blue you will see that there is a little different distribution in that the metachromasia tends to be much more aggregated about the cartilage cells themselves (Figure 5A) One can change this tendency by enzymatic digestion say, with trypsin or pepsin, so that the entire matrix loses the localized metachromatic stain (Figure 5B)[18] Now, whether this, as we are going to mention again indicates that the cartilage cell has something to do with the formation of this metachromatic material, I do not know

Hastings May I ask a question? Are the cells in Figure 5 normal cells after freezing? Have they been destroyed?

Follis They have shrunk some That is all I can say If you make a free hand slice, without freezing you get the same picture

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[18]Follis R H Jr Effect of Proteolytic Enzymes and Fixation on Metachromasia of Skin Collagen, *Proc Soc Exper Biol & Med* **76** 272 273 (1951)

Armstrong The spaces represent the areas occupied by the cells?

Follis Yes The black staining material is matrix, which stains more deeply than the material in between

Hastings Will the components take the stain *in vivo* before you kill the animal?

Follis I have never done that, but I would imagine that they would

We have been studying skin in scurvy and following cortisone treatment One way of observing skin is to study the shrinkage temperature This is something that has been used by the leather chemists for a number of years as an index of the adequacy of tanning, that is, if you put skin in water and heat it at 65 or 66° C, it suddenly contracts This is an interesting phenomenon to watch The preparation is set up in a modified Dale bath and recorded on a smoked drum (Figure 6) You can sit there, and as the temperature reaches 65°, you can say, "Contract," and the skin contracts!

Hastings Does it make any difference what pH the solution is that you put the skin in?

Follis It does if you go to extremes, but at neutrality, no Salts have different effects Formaldehyde and chrome, which is used in tanning, raise the temperature of contraction to over 100° C

Kochakian What is your definition of skin? That is, how deep do you go?

Follis It is the epithelium and the corium, without the fat

This phenomenon is thought to be due to disruption of hydrogen bonds, which hold the fibers extended, heating allows them to fold [19, 20] Skin which has been treated in this way just for your interest, is like rubber You can pull it out and it snaps back If one gets into the literature of the leather chemists, one finds that they have done a tremendous amount of work

Hendricks Is this change in the skin a permanent alteration?

Follis Yes Well, we thought we would try it with cartilage, just for fun, and I believe the results might interest you The cartilage contracts,

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[19] Weir, C E, and Carter, J Rate of Shrinkage of Tendon Collagen Further Effects of Tannage and Liquid Environment on the Activation Constants of Shrinkage, *J Am Leather Chemists' Assoc* 45 421-439 (1950)

[20] Theis, E R, Wilson, C D, and Halpin, H Protein Axial Movement Studies III Elongation and Contraction of the Collagen Axis by Means of Acids Bases and Heat, *J Am Leather Chemists Assoc* 45 610 642 (1950)

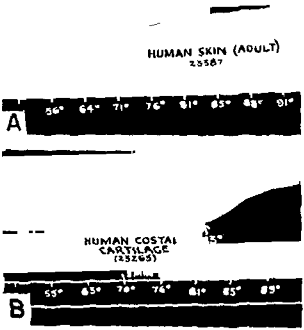


Fig 6 Comparison of the Effect of Heat on the Shrinkage of Human Skin and of Human Costal Cartilage

A Shrinkage temperatu e curve of normal skin which began to contract at approximately 66° C *Time* signal interval *2* minutes *B* Similar curve of a preparation of a slice of costal cartilage which contracted less precipitously and at a higher temperature than d d skin

but contracts at a much higher temperature What that means I haven t the slightest idea Perhaps one of you chemists will know Whether the difference is purely physical or whether it is chemical I don t know

The Chemical Structure of Bone

Like cartilage the organic component of bone (osteoid) has a fibrous structure when examined with the light microscope (Figure 7) The in organic components first must be removed by acid before this structure can be revealed by appropriate digestion technics The collagen fibers making up the organic phase of bone show a typical periodicity of approximately 640 A which you will recall Dr Robinson[21] showed us last year Collagen

[21]Robinson, R A Electron Micrography of Bone *Trans Macy Conference-on Metabolic Interrelations* 3 271 289 (1951)



Fig 7 Bone Treated to Bring Out the Structure of the Matrix (Osteoid)

Silver stain of bone to show fibers entering a trabecula They are obscured in the trabecula by the presence of the inorganic constituents

derived from bone after decalcification has the same approximate amino acid pattern as collagens from other sources [9] so that it is quite likely that what we have already said about the protein content of cartilage applies equally well to bone

Bone also is metachromatic which may indicate that chemically it is closely related to cartilage And of course bone protein has the same amino acid composition as does gelatin from cartilage skin and so forth

Armstrong To what in bone is the metachromasia due?

Follis Well I am going to get to that in just a minute The polysaccharide component of bone has not been studied at all extensively, that is we do not know whether or not there is chondroitin sulfuric acid in bone The sulfate content of bone is lower than that of cartilage [22] but hexosamine and glucuronic acid have been isolated from bone in appreciable quan

[22] Meyer K The Chemistry and Biology of Mucopolysaccharides and Glycoproteins *Cold Spring Harbor Symp Quant Biol* 6 91 102 (1938) edited by Ponder E published by the Biological Laboratory Cold Spring Harbor New York

tities [3, 4] This whole question of the composition of bone matrix has been tremendously retarded by the presence of the lime salts

Fremont Smith Do we know what causes metachromasia in any tissue?

Follis Well mucopolysaccharide is metachromatic hyaluronic acid is metachromatic and it is assumed by Dr Kurt Meyer (I don t know whether or not Dr Karl Meyer has data on this point) that chondroitin sulfuric acid is metachromatic

Karl Meyer The problem of metachromasia is still controversial As to the metachromasia of hyaluronic acid some state [5] it is metachromatic and others say it is not There is no controversy about the metachromasia of the chondroitin sulfates

Bartter May I ask a question? Can somebody explain briefly the chemical change involved in the transition from the red to the blue response?

Follis There is a shift in the absorption and the change in the color reaction is thought to be due to a polymerization or an aggregation of the dye molecule

Bartter What is the dye?

Follis Toluidine blue or methylene blue

It is of interest that uncalcified bone (osteoid) which is encountered in large quantities in rickets is metachromatic[26] Another organic material citrate is found in high concentrations in bone We cannot go into its possible significance which recently has been reviewed [27]

Well that about finishes what I know about the chemical structure of cartilage and bone

The Formation of Cartilage Matrix

Biochemical observations have revealed that metabolically cartilage is

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[23]a Rogers H J Concentration and Distribution of Polysaccharides in Human Cortical Bone and the Dentine of Teeth *Nature* **164** 625 626 (1949)

b Rogers H J The Polysaccharide Associated with the Organic Matrix of Bone *Biochem J* **49** xii xiii (1951)

[24]Logan M A Composition of Cartilage Bone Dentin and Enamel *J Biol Chem* **110** 375 389 (1935)

[25]Gross J Connective Tissue Fine Structure and Some Methods for Its Analysis *J Gerontol* **5** 343 360 (1950)

[26]Follis R H Jr and Berthrong M Histochemical Studies on Cartilage and Bone I The Normal Pattern *Bull Johns Hopkins Hosp* 85 281 296 (1949)

[27]Dixon T F Citric Acid and Calcium Metabolism *J Bone and Joint Surg* **33B** 268 271 (1951)

not a very active tissue It does not seem to be necessary to summarize the few studies which have already been recorded You will recall that Dr Gutman went into this two years ago [28] The cartilage cell contains neutral fat and an increasing amount of glycogen as it becomes more differentiated [6] The mechanism whereby the cartilage cell influences or promotes the formation of intercellular matrix is completely obscure As we have already noted if unfixed frozen sections are stained with toluidine blue augmentation of the metachromatic reaction is found about the cells Whether or not this indicates that polysaccharide if this be the cause of metachromasia is synthesized closest to the cell can only be conjectured How the molecules of glucuronic acid and acetyl galactose amine are polymerized is of course a complete mystery

The Formation of Bone Matrix

The formation of bone matrix is mediated in some unknown way by the osteoid forming cells the osteoblasts Before going on to a description of the possible mechanism of the formation of the bone matrix something must be said about the histochemistry of the bone forming cells In ordinary sections the cytoplasm of the osteoblast is characterized by its basophilia stained with pyronin this material is pink suggestive evidence that it is ribose nucleic acid (R N A) When ribonuclease is applied to bone sections the basophilia and pyronin staining material disappear, indicating pretty definitely that it is R N A [29] The osteoblast is rich in cytochrome oxidase (as evidenced by the reaction to the nadi reagent) and exhibits alkaline phosphatase activity [26] The cytoplasm of the osteoblast contains glycogen [26]

Shorr Has there been any demonstration of the presence of the cytochrome oxidase system in cartilage cells?

Follis The cartilage cells do not contain it and I don t know why This difference is one of the pieces of evidence of a considerable degree of dissociation between cartilage cells and osteoblasts In addition we have a number of examples in which you can injure the metabolism and function of the osteoblast without changing the function or the growth activity of the cartilage cell I am going to discuss one example in a minute

Shorr And yet there is some evidence that an oxidative type of metabolism does take place in cartilage

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[28] Gutman A B and Yu T F A Concept of the Role of Enzymes in Endochondral Calcification *Trans Macy Confer nce on Metabolic Interrelatio s* 2 167 190 (1950)

[29] Follis R H Jr Histochemical Studies on Cartilage and Bone II Ascorbic Acid Deficiency *Bull Johns Hopkins Hosp* 89 9 20 (1951)

Follis Cytochrome oxidase has been studied manometrically in cartilage slices that is not histochemically but actually chemically and has not been found

Shorr The evidence that we have obtained for an oxidative metabolism in the hypertrophic cartilage cell came from work done with Dr Howard Hiatt[30] using the reagent triphenyl tetrazolium chloride This substance is water soluble and colorless and has the interesting property of acting as a hydrogen acceptor for intracellular reductases Upon enzymatic reduction it is converted into a deep red insoluble material which is deposited intra cellularly presumably at the site of enzymatic reduction In functioning as a hydrogen acceptor it competes of course with other systems in the cell such as the cytochrome oxidase system which normally serves this purpose It is possible to favor one system or the other by varying the oxygen ten sion in the medium thus if we incubate a tissue slice in an atmosphere of oxygen the cytochrome cytochrome oxidase system is favored and there is very little reduction of tetrazolium On the other hand under anaerobic conditions the reduction of tetrazolium is maximal When slices of rachitic cartilage are incubated *in vitro* with tetrazolium in 100% oxygen there is virtually no reduction of tetrazolium If however the same procedure is carried out under completely anaerobic conditions there is an intense re duction the tetrazolium being deposited as the deep red insoluble formozan in the hypertrophic cartilage cells This means that some type of oxidative system must be operative in the hypertrophic cartilage cell However this does not mean that it is the cytochrome oxidase system

Follis It would be another oxidase

Shorr Yes

Follis That is true isn t it Dr Gutman?

Gutman Some other oxidases may be present

Follis But apparently cytochrome oxidase is not

Armstrong Is cytochrome present in cartilage tissue? One would not expect cytochrome oxidase to be present in a tissue unless the cytochrome system were present would one?

Follis No, I wouldn t think so I don t know but I don t think that has ever been studied

Gutman There is very little cytochrome present as determined by spec

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[30] Hiatt H and Shorr F Unpublished data

trophotometric measurement in cartilage according to Lutwak Mann [31]

Follis Of course like certain other tissues which contain large quantities of mucopolysaccharide such as cornea and umbilical cord cartilage is very avascular and its metabolism is distinctly not very high but it does have a definite metabolism as you have indicated

We have done some tetrazolium experiments also not paying careful attention to oxygen but it probably was not present We could not get any reduction unless we added succinic acid in which case a rather good reduction was obtained in apparently the right areas

Shorr The reduction of tetrazolium by hypertrophic cartilage can be clearly demonstrated in the absence of added succinic acid It is questionably intensified in the presence of succinic acid To measure this difference and hence to provide some quantitative indication of the amount of succino dehydrogenase activity in cartilage cells would require a quantitative procedure very difficult to employ with this tissue This procedure consists in the extraction with acetone of the colored formozan and measuring it in a colorimeter This can be done readily with soft tissue

Follis Of course cartilage does have succino dehydrogenase activity You can show that with slices

Shorr Oh yes

Follis I wasn t going to go into the metabolism inasmuch as it was covered here previously by Dr Gutman

Perhaps the most instructive material with which to study the formation of bone matrix may be derived from the early stages of healing scorbutus You will recall we illustrated this two years ago [3] As you will remember ascorbic acid deficiency is characterized by an inability of certain specialized cells (fibroblasts osteoblasts and odontoblasts) to influence or promote the formation of intercellular substances collagen osteoid and dentin Scurvy in the skeleton therefore is characterized by an absence of osteoid formation Spindle shaped cells which one must regard as impotent osteoblasts are present and are perfectly able to reproduce In their vicinity however there is *not a vestige of intercellular matrix* As soon as healing is instituted by the administration of vitamin C one encounters certain dramatic changes [28, 32]

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[31] Lutwak Mann C Enzyme Systems in Articular Cartilage *Biochem J* 34 517 527 (1940)

[3] Follis R H Jr The Influence of Essential Nutrients and Hormones on Cartilage and Bone *Trans Macy Conference on Metabolic Interrelations* 2 221 257 (1950)

Fine argyrophilic fibers (reticulum) appear in the vicinity of the osteoblasts These fibers are metachromatic and give a positive alkaline phosphatase reaction Certain changes also appear in the osteoblasts coincident with the appearance of the fibers Cytochrome oxidase activity which is decreased or absent in absolute scurvy returns as does the phosphatase activity (which also disappears as a result of vitamin C deficiency) In addition the cells become plump and engorged with R N A glycogen also appears in the cytoplasm

Can any sense be made of such changes? Briefly it appears fairly well accepted that R N A is associated with protein synthesis[33] So too phosphatase activity seems to be correlated with the elaboration of fibrous protein[34] The participation of cytochrome oxidase and/or glycogen in the production of fibrous protein is as yet not established There are thus at the moment some data which implicate the osteoblast and certain of its intracellular constituents in protein synthesis[35] We can go no further with regard to the formation of any other proteins One recent observation should be mentioned because as we noted above the newly formed fibers in healing scorbutus give a positive phosphatase reaction Gold and Gould[37] have found that phosphatase combines firmly with freshly precipitated fibers of rat tail tendon, they postulate that positive staining reactions in sections result from adsorption of the enzyme on the newly formed fibers This observation would still not negate the importance of phosphatase in the formation of fibrous proteins since the enzyme is reduced or absent in scorbutic tissues and does not reappear until vitamin C therapy is instituted[32]

The Destruction of Cartilage and Bone Matrix

Cartilage matrix is destroyed at the cartilage shaft junction after bone is deposited upon it Capillaries invade the columns of hypertrophic cells either because the cells have died or because the blood vessels kill them The spicules of calcified matrix encased in a thin layer of bone are then destroyed doubtless both by osteoblasts as well as osteoclasts The latter

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[33]Caspersson T The Relations Between Nucleic Acid and Protein Synthesis *Symp Soc Exper Biol No 1 Nucleic Acid* pp 127 151 (1947) edited by Danielli J F and Brown R Macmillan Co New York

[34]Bradfield J R G Phosphatase Cytochemistry in Relation to Protein Secretion *Exp Cell Res Supp* 1 338 350 (1949) edited by Caspersson T and Monne L Academic Press Inc New York

[35]These relationships have been recently discussed by Davidson[36]

[36]Davidson J N *The Biochemistry of the Nucleic Acids* John Wiley and Sons New York (1950)

[37]Gold N I and Gould B S Collagen Fiber Formation and Alkaline Phosphatase *Arch Biochem* 33 155 164 (1951)

cells would appear also *to destroy uncalcified* cartilage, since one may observe them on the undersurface of costal cartilage in cases of renal rickets associated with osteitis fibrosa

The exact mechanism of the destruction of osteoid is unknown The process is further complicated by *the fact that some mechanism also has to* be formulated for the simultaneous removal of the inorganic materials which have been deposited in the matrix Destruction is doubtless mediated in some way by cells, that is, osteoblasts and osteoclasts

When we come to the destruction of bone and cartilage, I think we draw almost a complete blank It is generally agreed—I think Dr McLean agrees with this—that osteoblasts and osteoclasts will destroy bone I can demonstrate to you that osteoclasts appear to be destroying cartilage There has been a great deal of discussion as to whether osteoclasts will *destroy osteoid* or *whether the* osteoid has to be mineralized but in our large series of cases of rickets Dr Park and I *have time and time again* seen osteoclasts destroying osteoid Furthermore, Dr Carnes[38] a year or so ago produced rickets in rats and then gave them large doses of parathyroid hormone and found that the osteoid was being actively destroyed by these cells In Figure 8A, there is an osteoclast right against cartilage, and the only evidence that one has that cartilage is being destroyed is the presence of these cells *This* is a case of *renal rickets in a child, in which there* is active destruction osteitis fibrosa In Figure 8B from a case with dietary rickets there is a large chunk of osteoid with just a little bit of mineral deposition and there are osteoclasts on either side

What do we know about the mechanics of destruction? I think, as far as I am concerned, we know nothing It must be enzymatic, or I think it is probably enzymatic, in nature We have been working on this problem for a little while using all sorts of different enzymes products from bacteria, and so forth If you take a piece of fresh, calcified bone, you cannot digest it unless you remove the minerals *If you remove the minerals you can* digest it The only other possibility we can visualize—and this is pretty far fetched—is that one of these cells might contain some chelating agent which would remove the mineral component, and then it would be able, with some proteolytic enzyme, to finish the process We use chelating agents to decalify bone, and that is why the thought struck me

When one comes to consider first the stimulus to the formation of osteoclasts, and, secondly the stimulus on this cell and the osteoblast, too, which leads to osteoid and bone salt destruction the problem becomes most

[38] Carnes W H The Demonstration of an Effect of Parathyroid, Extract on Bone Matrix *Am J Path 26 736* (1950)



Fig 8 Relationship of Osteoclasts to Destruction of Cartilage and of Osteoid

A Osteoclasts in apposition to costal cartilage in a case of renal rickets (osteitis fibrosa) in a child *B* Osteoclasts in apposition to osteoid (dietary rickets)

complex Parathyroid hormone would appear to effect bone destruction directly by cellular stimulation[39, 40] So too it is quite likely also that humoral concentrations of inorganic ions are extremely important[41, 42] It would appear probable that bone destruction is enzymatic in nature However can this explain the solubilization of bone salt No one to my knowledge has seen inorganic particles in the osteoclast It would appear unlikely that pH change could effect a solution of bone salt

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[39]Barnicot N A The Local Action of the Parathyroid and Other Tissues on Bone in Intracerebral Grafts *J Anat* 82 233 248 (1948)

[40]Chang H Grafts of Parathyroid and Other Tissues to Bone *Anat R* 111 23-39 (1951)

[41]Jaffe H I Bodansky A and Chandler J P Ammonium Chloride Decalcification as Modified by Calcium Intake The Relation between Generalized Osteoporosis and Osteitis Fibrosa *J Exper Med* 56 823-834 (1932)

[42]Gomori G and Gulyas E Effect of Parenterally Administered Citrate on the Renal Excretion of Calcium *Proc Soc Exper Biol & Med* 56 226-228 (1944)

I want to bring up one other extremely interesting observation again stemming from Dr Gross s laboratory and that is the effect of tension on the effectiveness of enzymatic activity Sizer[43] has shown that you can change by putting weights on sutures of tendon I believe the time that it takes for digestion by trypsin I think that is the only enzyme he studied This has some implications as far as bone is concerned because in the re modeling as a result of stress and strain there must be differences in ten sion That there will be differences then in the effectiveness of enzymatic activity is a real possibility

Fremont Smith Tension accelerates the rate? You said it changes but you didn t say in which direction

Follis Accelerates

I trust that this introduction will serve to indicate the many gaps in our knowledge regarding cartilage and bone matrix We are only beginning to make progress in our understanding of these materials

Conference Discussion

Armstrong You have certainly reviewed the field in your usual capable fashion Dr Follis I thank you very much I would like to invite spon taneous discussion now If the discussion does not develop in the way that I expect it to I will invite specific discussion I would like to know if there are any questions or brief comments

McLean Dr Follis did not bring out in his discussion any differences between a calcifiable and a non calcifiable matrix I hope that that will ap- pear in some of the discussion which will follow

Follis Would you elaborate on what you mean by that? What do you mean by non calcifiable matrix?

McLean Well cartilage matrix for instance Hyaline cartilage is as a rule not calcifiable Where intracartilaginous ossification is going on the cartilage matrix becomes calcifiable near the front at which bone is ad vancing into and replacing the cartilage

Follis However you can calcify it by artificial methods, that is, if you leave it in contact with concentrations of lime salts long enough they will deposit on it

McLean Most connective tissue can be calcified under very special con ditions but ordinarily skin and the matrix of hyaline cartilage do not calcify

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[43] Sizer I W The Action of Trypsin and Other Proteases on Collagen *Enzymol* 13 293 301 (1949)

Follis No, of course. Aorta has somewhat similar characteristics and so does cornea. You can calcify the aorta *in vitro*. Robison[44] did that or one of his associates did. If you leave it in contact with the mineral salts long enough under physiological conditions it will calcify.

McLean Referring to the special affinity for calcification that certain tissues have, there must be either some chemical or physical explanation for that which still does not appear.

Fremont-Smith In other words, the similarities are very important but the differences are also very important.

Follis Yes.

Johnson One point might be added. Unless my memory is serving me false, I believe that the contraction of tanned collagen applies to individual fibers as well as to skin, which, of course, is very important in a number of the questions that have been asked.

Follis That has been brought out with the electron microscope by W. J. [illegible]pers.[3] I don't know whether you have done any work on that, Dr. Gross.

Gross We haven't done very much, but I believe that the linkage between the fibrils themselves is more on a histological level and is important in tanning as well as the intrafibrillar penetration of the tan.

Follis Well, there are CH_2 linkages with formalin treated materials and there are chrome linkages which apparently make it very much more stable with chrome tanning.

Meyer But this contracture happens with untanned preparations.

Gross The heat causes contraction, yes. The heat contraction will take place with almost any type of collagen and there are many factors involved. Normal collagen, which is untanned and untreated and in a wet state, contracts at a relatively low temperature and various types of collagen contract at different temperatures. For example, the tendon collagen contracts at a lower temperature than hide collagen, and collagen from the swim bladder of the fish, for example, just goes into solution at about 40° without even contracting. The pH plays quite a part in lowering the shrinkage temperature, as do various other reagents, such as formamide, or anything else which swells the collagen.

Follis It is very interesting that calcium is unique among most of the cations in reducing the shrinkage temperature in solutions. Collagen practi-

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[44] Rosenheim, A. H., and Robison, R.: The Calcification *In Vitro* of Kidney, Lung and Aorta. *Biochem. J.* 28: 712-719 (1934).

cally contracts at room temperature in the presence of calcium ions

Hastings How much calcium is there in these solutions?

Follis The calcium was present in fairly high concentration I can t tell you Dr Hastings, in millimols

Choucroun At what pH?

Follis Normal pH

Choucroun Well, I think you could have it with less concentration if you were working in a pH which would be a little alkaline, because that would facilitate the fixation of the calcium ion on the cells Did you try that—to change the pH?

Follis I am not studying that phase of this phenomenon I will leave that for the leather people or the biochemists

Sobel But there are differences between collagens For instance, gelatin derived from fish skin collagen has an isoelectric point of the order of 9 4, whereas gelatin derived from bone collagen has an isoelectric point around 4 5

Follis Isn t it true Dr Hamilton, that the amino acid content of gelatin as derived from skin cartilage or bone is practically identical?

Hamilton I wish I felt competent to answer your question

Follis I think this observation is in the literature

Hamilton I feel certain that there is still work to be done to settle that question I would expect that the fish swim bladder gelatins would turn up with a slightly different composition from the ordinary gelatin as we know it because I would assume that it is probably not made under the circumstances that ordinary gelatin is made, which has an extensive liming treatment

Meyer As far as I remember, fish bladder collagen the so called ichthyocol is remarkably different from mammalian collagen in its amino acid composition The definition of collagen as given by the electron microscopists and by the x ray diffraction people, is purely a structural one and I think that ought to be made clear here

Follis Of course, fish collagen is in a different ionic environment than our collagen is

Gross Well actually, the collagens differ a good deal one from the other both physicochemically and chemically The analytical chemical difference of collagen in the mammals is very small, but the physicochemical

differences between one collagen and another may be quite large For example in the rat the skin behaves quite differently from the tail tendon Rat tail tendon will go into solution very rapidly in extremely dilute acid of a pH of 4 and below whereas skin collagen will swell only at pH 1 and still maintain its crossbanded structure Fish swim bladder collagen reacts to acid and subsequent salting very much like that from rat tail tendon yet the amino acid composition varies in some particulars When you go down to the sponges which give essentially the same wide angle x ray pattern as collagen from the mammals the amino acid composition is almost entirely different The amino acid pattern of sponges resembles that of an entirely different protein in fact there is very little proline or hydroxyproline—one of the distinguishing characteristics of mammalian collagen

Follis It is interesting that rat skin and guinea pig skin especially of young animals will contract and then will un contract as the temperature is raised still higher In other words as the temperature goes up the con traction decreases

Fremont Smith Is this response then reversible or is the whole process finished?

Follis I have suppo ed that it is reversible

Fremont Smith If you bring the temperature down again you can go through the same phases and obtain the same sequence again?

Follis Well we haven t done that

chain helix with a short pitch rather than the three chain helix with a lazy spiral Actually Pauling and Corey suggested some such similar model some years ago and then discarded it

The Periodicity of Collagen Fibrils

The work I would like to discuss does not deal directly with bone or cartilage but more with fibrogenesis and the formation of collagen which is perhaps related to the discussion of any connective tissue structures This work represents a joint effort of Dr F O Schmitt Dr J H Highberger and myself We have been at it for a number of years

The experimentation on reconstitution of collagen actually began with Nageotte[49] and Faure Fremiet[50] in France in the 1930 s They found that certain types of collagen are soluble in dilute acid for example rat tail tendon and swim bladder collagen which will dissolve producing a clear viscous solution at about pH 3 5 in almost any of the acids Upon the addition of salt or neutralization a fibrous precipitate appears These fibers histologically have a strong resemblance to collagen In the early 1940 s Schmitt Hall and Jakus[46] were able to show that the fibrils as produced by neutralizing such a solution have an axial periodicity very much like that of collagen—and I should at this point mention that the original observations of periodicity of collagen fibrils in the electron microscope were made by Hall Jakus and Schmitt[51] in 1942 and simultaneously by Wolpers[52] in Germany Bear[53] and independently Kratky and Sekora[54] just prior to this had demonstrated the periodicity in collagen by means of the x ray diffraction technic Since then it has been found that most forms of collagen which can be identified as such by the wide angle x ray pattern have an axial periodicity averaging 640 A that is all the known collagens of mammals fish reptiles birds on down through the Coelenterata[55] Recently *we examined the collagen of the sea cucumber with the*

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[49]Nageotte J and Guyon L Reticulin, *Am J Path* **6** 631-654 (1930)

[50]Faure Fremiet E and Garrault H The Acicular Connective Tissue of the Swim Bladder *Arch d Anat Micr* **33** 81 94 (1937)

[51] Hall C E Jakus M A and Schmitt F O Electron Microscope Observations of Collagen *J Am Chem Soc* **64** 1234 (1942)

[52]Wolpers C Collagen Connective Tissue and Ground Substance *Klin Wchschr* **22** 624 (1943)

[53]Bear R S Long X Ray Diffraction Spacings of Collagen *J Am Chem Soc* **64** 727 (1942)

[54]Kratky O and Sekora A The Detection of Large Distances Between Lattice Planes in Kangaroo Tail Tendons *J Makromol Chem* **1** 113 121 (1943)

[55]Marks M H Bear R S and Blake C H X Ray Diffraction Evidence of Collagen Type Protein Fibers in the Echinodermata, Coelenterata and Porifera *J Exper Zool* **111** 55 78 (1949)

electron microscope and found even there the typical periodicity of collagen. Also interestingly, Dr Schmitt several years ago found that the collagen from a 20 000 year old mammoth tusk also had this periodicity.

Follis Isn't that 640 figure an average?

Gross Oh yes, certainly. When we say 640 what we mean is the average of a distribution curve—a Gaussian type curve which varies from about 400 Angstroms up to about 1000, most of it falling in the range between about 500 and 700. The dispersion also varies somewhat with different tissues. The distribution curve for rat tail tendon, for example, is dispersed somewhat more than it is for skin. But the peak always falls around 640 or 650.

Figure 9 shows the typical picture of collagen fibrils from human skin.[56] This was prepared by freeze sectioning fresh skin corium and allowing the fragments to deposit on the collodion film from a suspension, and you can see that these are the fibers which could be seen histologically as a thin line. The fiber is made up of the individual fibrils, displaying the usual axial period. Rat tail tendon is different in that the fibril width varies tremendously and the fibrils also fray readily, whereas these fibrils are relatively uniform in width and rarely fray.

I show Figure 9 to indicate the detailed, intraperiod fine structure ranging from one to six bands. We have labeled them A, B_1, B_2, C, D, and E. The position of these bands is quite characteristic for all the mammalian collagen we have examined. Even in the fish swim bladder, where the amino acid composition is somewhat different, the banding is the same. When you stretch these fibrils, the sub periods increase in exactly the same proportion as does the total period.[57] We feel that this six banded structure is a very good fingerprint for the substance collagen. The fibril is polarized axially, i.e. it has a head and tail, which is an extremely interesting thing from the point of view of protein structure.

Follis Would you comment on drying? These are all dried specimens.

Gross Surely. In preparing specimens for the electron microscope, one has to dry them, the reason being that the column of the microscope is highly evacuated. The presence of gas molecules will scatter electrons and prevent image formation. These preparations are made by depositing an aqueous suspension of fragmented collagen on a very thin collodion film

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[56] Gross, J, and Schmitt, F O. The Structure of Human Skin Collagen as Studied with the Electron Microscope. *J Exper Med* 88 555-568 (1947)

[57] Schmitt, F O, and Gross, J. Further Progress in the Electron Microscopy of Collagen. *J Am Leather Chemists Assoc* 43 658-675 (1948)



Fig 9 Collagen Fibrils from Human Skin
Prepared by very gentle fragmentation of frozen section Magnification 30 000

covering a small disc of wire mesh Thin specimens are required because the electron beam will not penetrate a sample greater than about a tenth of a micron in thickness and give any sort of structural detail Drying actually has an effect which has been studied by us at M I T by means of a replica technic[56] One makes an imprint of wet collagen in a collodion film and examines that imprint in the electron microscope The corrugations are far less distinct than those in a replica of dry collagen X ray diffraction studies of Bear and Bolduan[58] also indicate that the fibril is smoothed out in the wet state The fact that the corrugation as seen in the electron microscope is a highly regular and uniformly reproducible artefact and that x ray diffraction patterns reveal the same periodicity in the whole wet native tissue demonstrates that the axial period is a real characteristic of the fibrous protein

There are two identifying characteristics of collagen which we feel are a little bit more definitive than the histologic picture namely the wide angle x ray diffraction pattern and this axial pattern in the fibrils seen with the electron microscope

Meyer The preparation in Figure 9 is stained?

Gross It is stained with phosphotungstic acid but the fine banding can still be seen in some unstained preparations as well It is just accentuated by the phosphotungstic acid

Follis Do you regard collagen then as a pure protein?

Gross It may be a mixture of proteins and perhaps other substances We just don t know at the present time

The fibrils in Figure 10 are from cartilage You can ignore the fine filaments in the background because they have nothing to do with the collagen The fibrils from the xiphoid process of the rat are essentially the same as those from skin

As I mentioned before collagen from skin differs in some ways from rat tail tendon When skin fibrils break they fracture without fraying When rat tail tendon fibrils break the ends are usually well frayed the individual strands of which still show the cross striations In dilute acid most of the human skin collagen fibrils just swell maintaining their axial period although there is some evidence that at least some of them dissolve Rat tail tendon collagen very largely goes into solution If such a viscous solution is filtered through sintered glass to make sure there are

and Salo T P A Model for Collagen Fibril X Ray Diffraction *J Am Leather Chem* ...

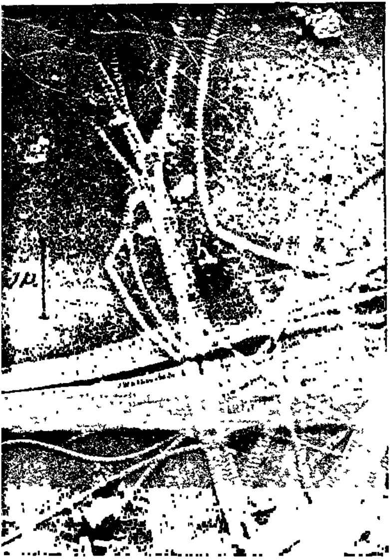


Fig 10 Collagen Fibrils from Rat Xiphoid Cartilage
Prepared by fragmentation and tryptic digestion Magnification 21 900 ×

no undissolved collagen fibrils present one obtains a picture such as is shown in Figure 11

The Effect of Enzymes on Collagen Structure

Follis Have you studied the effect of pepsin in modifying such a picture?

Gross Yes we have It is very difficult to know about pepsin because of the effect of acid

Follis Pepsin will produce changes at pH 5

Gross We haven t tried that We have worked with collagenase and hyaluronidase

Follis It would be interesting because as you know skin is not metachromatic[59] whereas all these other materials—tendon and cartilage and osteoid—are But if you digest it with pepsin at pH 5 it becomes metachromatic

Gross I am afraid I can t make much comment on that

Follis It might be that you could produce this fibrillar fraying by using pepsin

Gross It is possible We just haven t done it Our original experiments with pepsin were done at the optimum pH around pH 2 We examined the end products and were a little bit dissatisfied with our results We will probably go back to it some day since we are studying the effect of various enzymes We know for instance that trypsin does not seem to attack the collagen structure at all as far as we can tell with the electron microscope It might in some more subtle chemical way

Figure 11 is a photograph of a clear acid filtrate of ichthyocol but the findings are the same for rat tail tendon You can see very fine filaments all over the background We can t make out any structure We feel that maybe the structure is there but technical difficulties make whatever structure is present quite elusive at the moment We spent a lot of time beating our heads against that stone wall trying to find the characteristic cross banding in filaments less than 100 Angstroms in diameter and have had no success as yet The extremely low contrast of phosphotungstic acid stained filaments that are less than 100 Å in width and the interference by the structure of the collodion film in shadowed preparations have thus far defeated us It

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[59] Follis R H Jr Effect of Proteolytic Enzymes and Fixation on Metachromasia of Skin Collagen *Proc Soc Exper Biol & Med* 76 272 273 (1951)



Fig. 11 The Effect of Acid on the Collagen Ichthyocol

Prepared by dissolving in acetic acid at pH 5 and filtering through sintered glass Magnification 47,500 ×

is altogether possible that the characteristic structure will not be visible in such filaments under the best conditions

The Effect of Sodium Chloride on the Precipitation of Collagen Fibrils

If one repeats the experiment of Nageotte adding the clear filtrate to 1% sodium chloride one gets a fibrous precipitate These are the fibrils which comprise the precipitate in Figure 12 They have exactly the same periodicity as the collagen you saw originally

Armstrong Will you go over that again?

Gross Surely If one adds a clear filtrate in which there are no fibrils to a salt solution a precipitate occurs

Hastings How much salt?

Gross Ionic strengths of about 0 17 to 0 2 The fibrous precipitate that we get from the addition of salt to a final ionic strength of 0 17 consists nearly entirely of fibrils with the same cross striation and the same detailed fine structure of two to six bands that we had in the original material before it was dissolved in acid

Hastings Are the fibrils all separated?

Gross Yes they are They are separated just floating around in the solution You can sediment or filter out this mass of collagen for chemical or other analyses

Neuman Do these reformed fibrils have a head and a tail? (Laughter)

Gross Yes most of them have These fibrils come back polarized exactly as they were before The kinetics of this phenomenon of course is tremendously interesting to us We know also how to modify this structure a little bit

Hastings You haven t any moving pictures of one of them forming?

Gross No Electron microscopy is nowhere nearly developed to the point where continuous changes in solution can be studied The material must be dried *in vacuo* for study At the moment we are studying the process of fiber formation in stages to see if we can get some idea of the structural kinetics Vanamee and Porter[60] have published a paper recently on some of the stages of formation of collagen from solutions

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[60] Vanamee P and Porter K R Observations with the Electron Microscope on the Solvation and Reconstitution of Collagen *J Exper Med* 94 255 268 (1951)



Fig 12. *Fibrils With Typical Collagen Fibril 640 A Axial Period* Precipitated from a Collagen Solution

Prepared by adding a clear filtrate of collagen solution to 1% sodium chloride *Magnification 28,200 ×*

Choucroun Is the process reversible?

Gross Yes it is

Choucroun You can put it into solution again?

Gross Yes However each time you do it, you get less and less material precipitating out There is some change For example, we know that these fibrils swell more in water and are more soluble in water than the original collagen so there is some change, but structurally they come back identical with the originals as far as we can tell, and we feel that perhaps this has some implication in fibrogenesis *in vivo* Perhaps the cell secretes a building block in the form of a high molecular weight polypeptide chain or perhaps even a mixed high polymer precursor In the extracellular space the ionic atmosphere and perhaps other factors cause these building blocks to aggregate specifically to form a higher order of organization in the form of cross striated fibrils The *in vitro* experiment may be a model of such a process We have no evidence that enzyme activity is involved in the test tube reconstitution of collagen However we have not yet looked hard for such evidence Acid solutions of collagen are stable in the ice box for months, as indicated by their ability to reconstitute with salt

If one adds 2% NaCl (final concentration), one gets a somewhat different effect (Figure 13) Fibrils reconstitute, but they do not have a period of 640 Angstrom units, they have a period of about 220 Angstrom units The steps seem discontinuous There is no average period between these two You get either 640 or 220 Porter and Vanamee[61] found in some of their tissue cultures which produced collagen, some fibrils with a period of about 220 Angstroms Speaking just speculatively, perhaps the ionic or other factors in the environment of the culture medium is involved in determining whether one gets a fibrous structure with 640 Angstroms or with 220

If the salt concentration is increased much above 2 per cent there is a healthy precipitate, but there is no visible period in the fibrils (Figure 14) The salt concentration will determine to a great extent the type of structure

Hamilton May I ask one question? Is there any difference in the time which must elapse before these fibrils form as the result of the addition of solutions of different strength? Is it very rapid or is it slow?

Gross Well this is usually a very rapid phenomenon There is something peculiar involved in this connection, which I should insert here For

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[61] Porter K R, and Vanamee, P Observations on the Formation of Connective Tissue Fibers *Proc Soc Exper Biol & Med* 71 513 516 (1949)



Fig. 13. Fibrils With [illegible]0 A Axial Period Precipitated from a Collagen Solution

Prepared by adding a clear [illegible] collagen solution [illegible] sodium chloride. Magnification 31,[illegible]00 ×

a period of several years we were able to add our filtrates to salt solutions and get an immediate fibrous precipitate without any trouble, and then slowly, over a period of time, each new preparation we made took more and more time to precipitate—under exactly the same conditions—until finally we were unable to get precipitation—immediately following addition to salt and for a long time now we have not been able to get these immediate precipitates We have to dialyze in the salt to do it Recently some of our filtrates have been precipitating after standing in salt solution for about a week We know, for example with certain salts such as oxalate we can get an immediate precipitate, but there has been a change in our precipitability with sodium chloride which we don t understand at all However we continue to get perfectly good reconstitution by dialyzing in the salt

Choucroun Is this reversible also?

Gross This fibrous precipitate will go back into solution

Choucroun And when reduced to 1% salt what happens?

Gross If you are referring to the structureless fibrils produced with high salt concentrations and redissolved in acid a 1% NaCl precipitate of this solution is very small

Choucroun No, but do you get the same result as is produced with 1% salt?

Gross You get back some fibrils with a striation of 640 Angstroms

Meyer Have you done any x ray diffraction studies on this amorphous or non banded fibril?

Gross No we have not because there is a problem in orientation here Unless your material is extremely well oriented you cannot get a good small angle diffraction pattern We would probably get the wide angle pattern, but you would expect that because you can get a good collagen wide angle pattern even from gelatin We are more interested in the chemistry at the moment We expect to do this eventually

The Extraction of Collagen with Acid Buffers

Another phase of the problem in which we became very much interested several years ago was the effect of extraction of collagenous tissue with acid buffers of relatively high ionic strength as described by Orekhovitch and colleagues[62 63] They found that organic acid buffers such as citrate

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[62] Orekhovich V N Tustanovskii A A Orekhovich, K D and Plotnikova N I The Procollagen of Hide *Biokhimiya* 13 55-60 (1949)

[63] Tustanovskii A A On the Proteins of the Skin *Biokhimiya* 12 285 290 (1948)

of ionic strength around 0.2 at pH 3 to 4 would extract something from connective tissue which formed a viscous solution and precipitated as fibers when dialyzed against water. They called it 'pro-collagen.' *This is different* from the Nageotte experiment in that dialysis of an ordinary dilute acetic or hydrochloric acid solution of collagen against water produces a clear *transparent gel.* *Highberger, Gross* and *Schmitt*[64] showed that dialysis of a citrate extract against water produces a fibrous precipitate which when examined with the electron microscope reveals two different structural forms. One is characteristic of collagen, but the other has an axial period averaging 2200 Å with a complex intraperiod fine structure quite different from that of collagen. Frequently one sees individual separate segments or a small number of segments joined end to end to form short fibrils. We have called this structure 'long spacing' for want of a better name. We have not yet found it in native tissues and believe it to be a synthetic product.

The Effect of Glycoproteins on the Precipitation of Collagen Fibrils

Because extensive extraction of the tissue with alkaline phosphate buffer prior to making the citrate extract appears to prevent the formation of 'long spacing' fibers, and phosphate is well known to extract mucoproteins, we thought it might be interesting to add mucoprotein to our collagen solutions. For this and other reasons, serum glycoprotein as described by Weimer, Mehl and Winzler[65] and also Schmid[66] was added to an ichthyocol filtrate, which mixture upon dialysis produced a precipitate of 'long spacing' fibrils.[67]

If you dialyze an acetic acid solution of collagen against water, you get a clear, structureless gel and you can see no structured fibrils at all, just very fine filaments matted together—what you would expect a *gel structure* to be.

Hastings: Dialyzed collagen?

Gross: This is the collagen solution alone dialyzed against water. If

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[64]Highberger, J. H., Gross, J., and Schmitt, F. O.: Electron Microscope Observations of Certain Fibrous Structures Obtained from Connective Tissue Extracts. *J. Am. Chem. Soc.* 72: 3321-3322 (1950).

[65]Weimer, H. E., Mehl, J. W., and Winzler, R. J.: Studies on the Mucoproteins of Human Plasma. V. Isolation and Characterization of a Homogenous Mucoprotein. *J. Biol. Chem.* 185: 61-568 (August 1950).

[66]Schmid, K.: Preparation and Properties of an Acid Glycoprotein Prepared from Human Plasma. *J. Am. Chem. Soc.* 72: 2816 (June 1950).

[67]Highberger, J. H., Gross, J., and Schmitt, F. O.: The Interaction of Mucoprotein with Soluble Collagen. An Electron Microscope Study. *Proc. Nat. Acad. Sc.* 37: 286-291 (1951).

to an acid filtrate one adds serum acid glycoprotein in dilute acid solution nothing happens But upon dialysis against water a fibrous precipitate appears Highly purified serum glycoprotein in concentrations of 0 05 to 0 1 per cent, in a collagen solution containing about 0 05% protein when dialyzed against water produces nearly all long spacing fibrils (Figure 15) The large period and intraperiod fine structure is identical to that produced from citrate extracts alone Concentrations of about 0 01 to 0 03% serum glycoprotein produce mixtures of long spacing and collagen type fibrils (Figure 16) If the concentration of glycoprotein is lowered to 0 005 to 0 001% glycoprotein only collagen type fibrils are formed (Figure 17) These collagen type fibrils differ from those produced by salt reconstitution in that they do not appear to swell or dissolve in water

The Effect of Hyaluronic Acid, Chondroitin Sulfate and Heparin on the Precipitation of Collagen Fibrils

Several different samples of hyaluronic acid chondroitin sulfate and heparin when added to collagen solutions in various concentrations produced an immediate fibrous precipitate but no axial periodicity has been found Dialysis of such preparations against water has not changed them One preparation of highly purified chondroitin sulfate from cartilage kindly furnished us by Dr Karl Meyer produced on addition to collagen solution an immediate precipitate which contained characteristic long spacing fibrils but never any collagen Neither was dialysis required nor did dialysis produce any further changes

Chondroitin sulfate one of Dr Meyer s preparations by itself dissolved in water and deposited on a grid produces only a drying pattern The material is somewhat fibrous but you do not see any organized structure The addition of this chondroitin sulfate to an acid solution of ichthyocol produced a structureless fibrous mass (Figure 18 top) but in addition some areas showed typical long spacing fibrils (Figure 18 bottom)

Conference Discussion

Hastings These are 2200 Angstrom units?

Gross Yes

Armstrong Is it possible that the precipitation occurs so quickly that the usual result is an ill defined fiber Is it conceivable that some time would be required for the spacing to develop

Gross It is possible

Hastings Have you lowered the temperature



Fig 15 'Long spacing' Fibrils Precipitated from a Collagen Solution

Prepared by dialyzing against water a mixture of 0 06% serum glycoprotein in a collagen solution Magnification 28 800 ×



Fig. 16 Mixture of 'Long-s[
tated from a Collagen Soluti

Prepared by dialyzing against
collagen solution Magnification

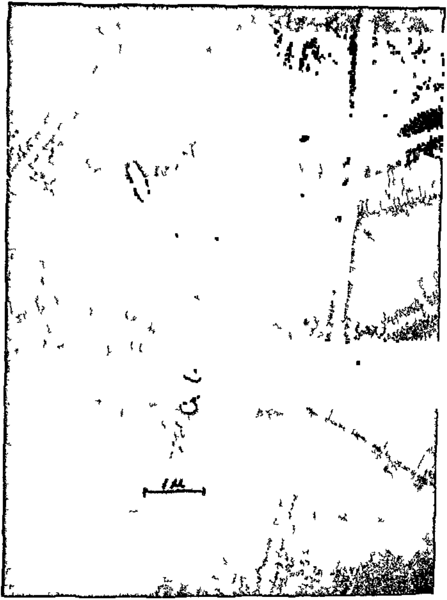


Fig 17 Collagen type Fibrils Precipitated from a Collagen Solution

Prepared by dialyzing against water a mixture of 0.001% serum glycoprotein in a collagen solution Magnification 16,600 ×

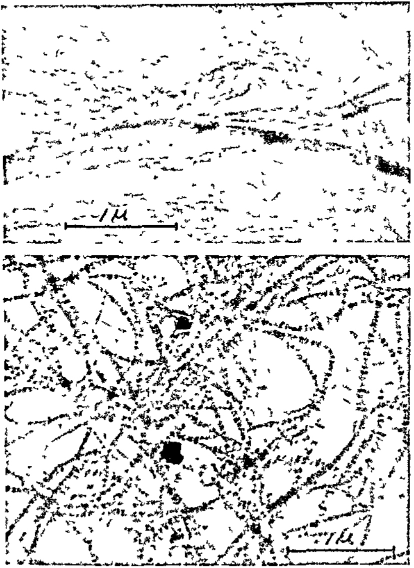


Fig. 18. Structureless Filaments and Long Spacing Fibrils Precipitated by Adding Chondroitin Sulfate to a Collagen Solution

Top. Structureless filaments (magnification 30,000 ×). *Bottom.* Long spacing fibrils (magnification 29,000 ×). Both photographs from the same grid which was prepared by adding a highly purified preparation of chondroitin sulfate from cartilage (prepared by Karl Meyer) to an acid solution of the collagen ichthyocol.

Gross We tried it at different temperatures yes *There is an important* temperature factor It is an ill defined one at the present time and we hope that when we get to studying the kinetics of this reaction we will learn more about the temperature

Neuman Were the different chondroitin preparations all salts of the same cation?

Gross I think that Dr Meyer knows They were calcium salts weren t they?

Neuman All three were calcium salts?

Meyer I really don t know what we sent Dr Gross I don't know the numbers I know the number of the preparation shown in Figure 18 but the two others, the negative ones which did not produce spacing I don't know what they were

Gross I think they were cartilage chondroitin sulfate, but I don t remember exactly what salts they were, either

Meyer The preparation in Figure 18 is a calcium salt, I know

Follis Of course you have found several different chondroitin sulfates?

Meyer They were calcium salts The material which gave a positive result was prepared from tracheal cartilage but so were others, which did not give the 'long spacing' The positive material was much more fibrous than the others

Hastings I was just going to ask if you would have obtained this result *if you had a high concentration of calcium*

Gross I don t know

Hastings Without it?

Gross No, calcium by itself has not given this result

Howard Have you ever studied any pathological material?

Gross Yes, we have We examined some material from rheumatoid nodules fibrinoid present in the knee joint on the synovia, and also some *skin from scleroderma, from cases of lupus erythematosus* disseminatus and from radiation burn, and the collagen that you see, that is the material that you can identify as collagen, seems to be structurally normal There is a lot of amorphous material in such preparations, but there is also in normal preparations I don't feel that the examination of pathological collagen with the electron microscope thus far, has been very revealing Gale[68]

[68]Gale J C Electron Microscope Studies of Collagen from Normal and Diseased Tissues, *Am J Path* 27 455 475 (1951)

and also Astbury's group[69] have described some studies on so-called 'collagen diseases' but the results are difficult to interpret

Follis Is citrate the only material that acts positively? As an extractor I mean

Gross No we have found that many of the organic acids and some of the inorganic acids if one increases the ionic strength with the homologous salt to about 0 2 extract material which will precipitate on dialysis against water to form both the 'collagen type and the long spacing type fibril

Meyer Have you tried solutions of hyaluronic acid?

Gross We have used different hyaluronic acids and serial dilutions

Meyer I mean as an extractant instead of citric acid?

Gross Oh to extract with hyaluronic acid?

Meyer Yes

Gross No we have never tried that One objection would be that extraction is done at acid pH and hyaluronic acid immediately precipitates with collagen

Meyer But it would precipitate only at low salt concentration At higher salt concentrations it would not precipitate

Gross Around 0 2 do you think it would?

Meyer No I don t think it would

Gross We just haven t tried that experiment There are so many ramifications in this thing We have tried to push along the line which would give us most definitive answers I have just been asked by whisper whether we can explain the entire phenomenon (Laughter) Actually we have a lot of hazy speculations about it but they are so numerous and so ill-documented that we haven t tried to present our ideas as to what we think is really going on here There are several questions of course that we would like to answer One of them is what is the chemistry involved? What is the analytical chemistry of these various substances? What is collagen What are the kinetics involved here? What sort of interaction has taken place? And of course what is the relationship of this phenomenon to what takes place *in fibrogenesis in the body*? We *don t* have definite answers yet

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[69]Kellgren J H Ball J Astbury W T Reed R and Beighton E Biophysical Studies of Rheumatoid Connective Tissue *Nature* 168 493-494 (1951)

Hastings Does it make any difference as to the right spacing whether or not you seed it with a little collagen?

Gross That is a good question, Dr Hastings, and, actually it was brought up only this past week, but we haven't got to it You are referring to the way insulin for example will seed

Hendricks As a member of the audience, I would like to raise a very speculative question and you can throw it down Do you have any speculation as to whether the smallest fibers that you obtain (in which the material is in its most dispersed form) reconstitute side by side or whether they wind and come down as pancakes? I think a member of the audience can speculate or raise such a question What do you say? Is there any evidence one way or the other?

Gross One might hypothesize a kind of zipper action We have pictures of fibrils forming in which the filaments may be seen fanned out from a roughly common central point with the characteristic axial period in nearly perfect register all across the fanned out fibril It would seem that the highly asymmetric building blocks come together at one point somewhere and then just zip right on up, and others are added to them But this again is sheer extrapolation We have no proof

Hastings Where do you think the tab is? (Laughter)

Hendricks Would you mind if I made one terminal comment about the structure of protein?

Gross Please do

Hendricks I have discussed this question of the structure of proteins with Dr Pauling and am encouraged to comment because of Dr Fremont Smith s earlier remarks There are arguments as to whether or not the currently suggested structures for proteins are valid but irrespective of this Dr Pauling made the striking advance of emphasizing that a few principles of molecular arrangement are basic to protein structure He recognized more clearly than did any one else that proteins have *structures*

Very much earlier a question had been asked about the nature of silicates and at that time few could grasp the idea that silicates have a systematics to them It is this matter of systematics the question of the generality as emphasized by Dr Fremont Smith that is important

Since proteins have structures they probably have only a few simple determinants for their structures Dr Pauling[70] recognized that one of

[70]Pauling L. Corey R B and Branson H R The Structure of Proteins Two Hydrogen Bonded Helical Configurations of the Polypeptide Chain *Proc Nat Acad Sc* **37** 205 211 (1951) Also seven following articles by Pauling L. and Corey R B *ibid* **37** 235 285 (1951)

these simple determinants is the pattern of the hydrogen bonds Now he can be *wrong about the particular patterns that he has suggested but these* can be found When they are found, the structures of most proteins will be analyzed just as have those of complex silicates But the step forward could not be made until one recognized that proteins really have structures The point is a philosophical one, one has to be wise enough first of all to ask the question, then the answer falls out

Follis You imply, then that the amino acid pattern will be kn wn

Hendricks Not entirely, at first, only the backbone patterns of the *polypeptide chain will be found These rather than the configurations* of the amino acid residues that stick out in various directions are important

Gross I think that those R groups that stick off must to a certain extent modify the pattern

Hendricks They do yes

Follis I was hoping you would say something about that because isn t that being studied in your laboratory with various procedures to change this x ray diffraction pattern?

Gross Yes Dr Bear has been doing a good deal of work on the structure of the various fibrous proteins by means of x ray diffraction One of the best ways to get information is first to get your standard pattern and then modify your material and find out how it changes under the influence of known reagents and known conditions A good deal of that sort of thing is being done

Johnson Is it speculatively possible that the oriented filament which you see when you use albumin and hyaluronic acid instead of serum polysaccharides *might be a midpoint in the change of phase between the clear solu*tion and the oriented pattern that ordinarily occurs so quickly that it is not observed?

Gross It is very possible that reaction rates play some role But as I say thus far we have no information about the kinetics here and although such a factor may be involved we feel it is more likely that the configuration of the molecules is of prime importance

Meyer In other words do you think if it is just a gel a fibrous meshwork there is a random distribution of units? If you use the proper agents do you *get an oriented structure, a repeating pattern?*

Gross I assume you mean reagents There may be a number of different conditions under which the collagen structure may be formed, sibly because the collagen molecules are more predisposed to line

proper register because of their specific structure However certain reagents such as the serum glycoprotein and a number of electrolytes in a particular range of ionic strength produce far larger numbers of clean highly specifically structured fibrils than you would expect from a random collision effect

Johnson Have you by any chance done x ray diffractions on long spacing fibrils?

Cross Yes we have Some preliminary wide angle diffraction studies have given the usual collagen wide angle diffraction pattern but the interpretation is not yet clear

Meyer Didn t Porter observe long spacing fibrils?

Cross No The fibrils with periods of about 1100 Angstrom units which he observed in tissue culture do not seem related to our long spacing fibrils at least not structurally[71] He does not believe they are directly related either

Armstrong I was very glad to hear Dr Hendricks make his comment He indicates that judicious imagination is required for progress I am reminded of a former teacher of mine who frequently said You will never make a diagnosis unless you think of the disease and you will not think of it unless you know that the disease exists Well what you are saying is that progress in this field is facilitated when one adopts the concept that structure does occur in proteins What the structure is remains to be worked out

Hendricks Pauling[0] himself refers to it as a stochastic method of intelligent guessing

Copp I would like to ask a question What is known of the interfibrillar substance in the bone matrix? So much is known concerning collagen and so little is known of the ground substance

Armstrong Protein other than collagen?

Copp Yes The collagen is common to almost all connective tissues so that there must be something else in the osteoid matrix which accounts for calcification

Follis Well you can get albumins and globulins out of skin Apparently you cannot get albumins and globulins out of cartilage at least as far as I know so there are other proteins in skin besides this protein collagen The leather people have studied that

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[71] Porter K R Personal communication

Gross When you say albumins and globulins do you mean the same kind that are present in the serum, or something different?

Follis I don't think they have been characterized

Copp In the interfibrillar ground substance of bone and cartilage is the protein different from collagen or is it a different form of the same protein?

Meyer Perhaps I can give a partial answer to that question

optimal or necessary Recently, Einbinder and Schubert[75] published a paper on the extraction of cartilage by various methods Calcium chloride was found to extract more collagen than did KCl If the protein extracted by $CaCl_2$ is collagen, then either the collagen of cartilage differs in its solubility from that of other collagens or the high concentration of chondroitin sulfate increases the solubility of the collagen

Gross Partridge[76] published a paper sometime ago in which he described the extraction of cartilage with hot water, above what he thought was the shrinkage temperature of the collagen present, and he came to the conclusion from electrophoretic studies of the extract, that he had three fractions One of them was polysaccharide, the second of them was gelatin and the third was a complex between the polysaccharide and the gelatin fraction But the only characterization he had of the gelatin was the isoelectric point and the electrophoretic mobility and he did not publish any analytical figures on the amino acid composition However, he felt that chondroitin sulfate was bound to what he called dispersed collagen in the cartilage

Dziewiatkowski You have found, Dr Meyer, that it is more difficult to get the polysaccharide out of skin than it is from cartilage?

Meyer Yes, with $CaCl_2$ solution it is practically impossible to get the polysaccharide out of the skin that is, the polysaccaride carbohydrate complex, while from cartilage you can almost quantitatively extract the polysaccharide with this $CaCl_2$ solution

[75] Einbinder J and Schubert M Separation of Chondroitin Sulfate from Cartilage, *J Biol Chem* 185 725 730 (1950)

[76] Partridge S M T[illegible] Com[illegible] I The State of Combination of Chondroitin Su[illegible] *Bio*[illegible] 397 (1948)

THE POLYSACCHARIDES OF MESODERMAL TISSUES[1]

KARL MEYER

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York

Armstrong: Dr. Meyer has consented to open the discussion this afternoon. I don't know what he will say but I think that he will have something to present with regard to chondroitin sulfuric acid, mucoprotein, and hyaluronidase. We will be pleased to hear anything he wishes to present.

Meyer: My comments will be some rambling remarks, since I didn't expect to say much here and had come to learn and to listen.

For many years our laboratory has been working on the ground substances of mesodermal tissues, or more specifically, with the polysaccharides contained in these tissues, especially hyaluronic acid and the chondroitin sulfates. This field has branched out very considerably in these years. Unfortunately, there are many facets in this field in which I am certainly no expert. For example, I would like very much to know more about histology, since a great deal of our information is based on histological studies using metachromatic staining, mentioned by Dr. Follis, and so forth. As chemists, we take a tissue, grind and extract it. Where the substances which we isolate were located and what their function is, we can only guess.

The Nature of Metachromasia

Right here I may say that the problem of metachromasia is very complicated. From Lison[2] we know that a substance has to have a high molecular weight and acidic groupings in order to stain metachromatically under the conditions defined by him. Lison thought that this requirement was fulfilled only by sulfuric esters, such as chondroitin sulfate, heparin and the mucoitin sulfate of the cornea and the gastric mucosa. More recently it has been pointed out that substances do not have to possess a sulfate group in order to be metachromatic. Metaphosphate has been shown to be metachromatic, and one of the strongest followers of Lison, Sylvén, of

[1] The experimental work reported in this paper was supported by the Josiah Macy, Jr. Foundation, the Helen Hay Whitney Foundation and the U. S. Public Health Service.

[2] Lison, L.: *Animal Histochemistry, Methods and Problems.* Paris, Gauthier-Villars (1936).

Stockholm has recently stated that oxidized cellulose shows alcohol resistant metachromasia In oxidized cellulose a varying number of glucose units are converted to glucuronic acid units, which would be responsible for the metachromasia

Whether or not hyaluronic acid is metachromatic has been debated quite considerably We found hyaluronic acid of a high degree of polymeriza tion metachromatic when tested in a concentration of about 1 per cent If the solution is depolymerized with hyaluronidase, the metachromasia dis appears almost instantaneously

I make these remarks in order to start a discussion on metachromasia I wonder for example about the quantity of a compound which has to be present in order to be seen by the histologists Dr Follis said that skin is not metachromatic but becomes metachromatic when treated with pepsin There is no doubt about the presence of both hyaluronic acid and a chon droitin sulfate in normal skin Why it is not metachromatic but becomes so on treatment with pepsin I do not know

The Nature of Chondroitin Sulfates

Now a few words about chondroitin sulfates Until a few years ago it was generally believed that there was only one chondroitin sulfate first characterized by Schmiedeberg in 1891 In 1941 we isolated from pig skin a chondroitin sulfate which had a rotation different from that isolated from cartilage namely −50° as compared with −30° for the latter Fur thermore while chondroitin sulfate of cartilage was hydrolyzed by testic ular hyaluronidase the compound isolated from skin was resistant to the action of the testicular enzymes From these observations it is obvious that the chondroitin sulfate of skin must be chemically different from that of cartilage

The structure of chondroitin sulfate of hyaline cartilage is not known al though my distinguished namesake Kurt H Meyer of Geneva has pub lished[19] a structure based on periodate oxidation Dr Follis has presented in Figure 4 the formula as proposed by Dr K H Meyer There are two glucosidic linkages in chondroitin sulfate, one hexosaminidic the other glucuronidic The same situation applies to hyaluronic acid Both glycosidic linkages are placed in the 1 3 position The configuration of both is β and both rings are assumed to have a pyranose configuration The sulfate is placed at carbon 6 of the N acetyl chondrosamine The two alternating components are assumed to be polymerized in a linear polymer of a molec

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[19] Meyer K H Odier M E and Siegrist A E The Constitution of Chondroitin Sulfuric Acid *Helv Chim Acta* 31 1400 1419 (1948)

ular weight estimated from hypoiodite titration as about 30 000 and from physical measurement[79] as about 300 000. There are so many unproved assumptions in the proposed formula that the suggested structure is highly questionable, especially since one of the most important points in the argumentation is not reproducible, that is, the hydrolysis of the sulfate group. By the way, periodate oxidation has led three different authors, among them K. H. Meyer, to propose three different structures for hyaluronic acid. Chondroitin sulfate of hyaline cartilage may be a branched chain polysaccharide. In our laboratory recently Mr. Linker has been studying the enzymatic hydrolysis of chondroitin sulfate by testicular hyaluronidase. The results so far obtained would be compatible with a branched chain molecule. The hydrolysis of chondroitin sulfate by purified testicular enzyme leads to various oligosaccharides. The end group of these oligosaccharides is the hexosamine. The glucuronidic bonds in the terminal positions are split by β-glucuronidase.

What I have said up to now applies only to chondroitin sulfate A of hyaline cartilage. About the other chondroitin sulfates we have very little information indeed. When we extracted connective tissue like heart valves, tendon, or umbilical cord, we found in all of these tissues at least two different polysaccharides. They are either hyaluronic acid and a chondroitin sulfate, as in umbilical cord, or two different chondroitin sulfates, one with a rotation of $-50°$, which we called chondroitin sulfate B, the other with a rotation of about $-20°$, which we called chondroitin sulfate C. Chondroitin sulfate B is resistant to testicular hyaluronidase and chondroitin sulfate C is hydrolyzed by this enzyme. In cartilage itself, that is, in tracheal cartilage, we also find two different chondroitin sulfates, A and C, distinct by their different solubilities and optical rotations.[80] We do not know the chemical basis of these differences, nor do we know anything about the topography of the compounds in connective tissue and their biosynthesis.

Follis: What interpretation can you make about these differences in optical rotation? Do they tell you anything about the form of the molecule?

Meyer: No.

Follis: They are just numbers.

Meyer: They are just physical constants. You see, there are a great many reasons for differences in the optical rotation of a polysaccharide. The linkages could be α or β. The uronic acid present may not be all glucuronic acid, or the amino sugars may be in part glucosamine and in part galactosamine, and so on. One might expect, further, that molecules of this

[80] Meyer, K., and Rapport, M. M.: The Mucopolysaccharides of the Ground Substance of Connective Tissue. Science 113: 596-599 (1951).

size would have faults that is non repetitive units in their structure though it would be meaningless to speculate about this

Polysaccharides and Protein Complexes

There is another point I want to take up here about which I talked this morning It concerns the protein complexes in which the mucopolysaccharides occur in the tissues We have gone on record that hyaluronic acid occurs not bound to protein but as a freely dissociated compound at least when it occurs in fluids such as synovial fluid or tumor fluids This conclusion is mostly based on the fact that electrophoretically at neutral reaction hyaluronic acid moves independently of the protein present in the fluid with a mobility identical for the isolated polysaccharide and the polysaccharide as it occurs natively in the fluids

Follis How do you determine that mobility?

Meyer You measure it in one of the commercial models of electrophoresis apparatus by photographing the zones There are several methods for determining the mobility of substances in an electric field They depend mostly on differences in refractive index

Fremont Smith You measure the difference in the rate of movement resulting from an imposed electric charge is that right?

Meyer Yes at constant temperature pH ionic strength and so forth

Armstrong The mobility is the same with or without the presence of protein?

Meyer Yes with hyaluronic acid

Recently however Ogston[8] in Oxford has concluded that hyaluronic acid occurs in synovial fluid as a protein complex I don t want to go into that in detail because we are concerned here more with cartilage and bone In cartilage as I said this morning we believe also that the major portion of the chondroitin sulfate occurs as a dissociable polar complex the cations being protein or any other cation present If I recollect correctly Blix reported that water extracts of cartilage on electrophoresis show the mobility of a fast fraction corresponding to that of isolated chondroitin sulfate If the chondroitin sulfate were in stable bond with protein the complex would have a slower mobility Furthermore with $CaCl_2$ solution one can extract the major portion of mucopolysaccharide from cartilage and obtain it free of protein by relatively mild procedures We have to admit however that

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[8] Ogston A G., and Stanier J F On the State of Hyaluronic Acid in Synovial Fluid *Biochem J* 46 364 376 (1950)

the evidence for the presence of chondroitin sulfate as an easily dissociable protein complex is weaker than it is for hyaluronic acid. On the other hand, no good evidence has been brought forth by which the existence of a specific chondromucoid can be established.

The Effect of Enzymes on Metachromasia

There is another point which I should have discussed in the beginning, namely, the interpretation of data on metachromatic staining of tissues, especially as it applies to cartilage. Recently, R. D. Lillie of the National Institute in Bethesda has published[92] some data on the disappearance of metachromasia following treatment with commercial testicular hyaluronidase and with amylase preparations. Upon incubation with either fresh or boiled solutions of these materials, the metachromasia of cartilage slices or of the basal membrane of arteries disappears. The agent responsible for this change in metachromatic staining has been called a chondromucinase.[92]

Gross: Won't trypsin do the same thing?

Meyer: Yes, trypsin will do this in some tissues, but the enzyme of Lillie was heat stable to a prolonged boiling, a treatment trypsin would not be expected to withstand. Maybe there was a proteolytic enzyme in these enzyme preparations of Lillie.

Gross: What I am referring to is this: just because the metachromasia disappears it doesn't mean that the agent producing the metachromasia has been destroyed. It may be simply that the linkage to the tissue has been destroyed and the metachromatic material has been washed out.

Meyer: That is possible. Another possibility is that these preparations contain a sulfatase. However, purified or crude hyaluronidases do not *in vitro* split sulfate from chondroitin sulfate.

Japanese authors[93] showed that there are different kinds of sulfatases, especially phenol sulfatases and glucosulfatases. The phenol sulfatases are found in animal tissues and in plant material.

Follis: Taka-diastase contains a sulfatase, as is well known, and it doesn't take out metachromasia.

Meyer: Taka-diastase contains a phenol sulfatase. That is different. The phenol sulfatases do not attack sulfate esters of carbohydrates.

Follis: Well, that is the only substrate that has been used, isn't it?

[92] Lillie, R. D., Emmart, E. W., and Laskey, A. M.: Chondromucinase from Bovine Testes and the Chondromucin of the Umbilical Cord. *Arch. Path.* 52, 363-368 (1951).

[93] Soda, T.: *Sulfatases. Methods of Enzyme Research.* Leipzig (1941).

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[81]Ogston A G and Stanier J F On the State of Hyaluronic Acid in Synovial Fluid *Biochem J* 46 364 376 (1950)

the evidence for the presence of chondroitin sulfate as an easily dissociable protein complex is weaker than it is for hyaluronic acid On the other hand no good evidence has been brought forth by which the existence of a specific chondromucoid can be established

The Effect of Enzymes on Metachromasia

There is another point which I should have discussed in the beginning, namely the interpretation of data on metachromatic staining of tissues, especially as it applies to cartilage Recently R D Lillie of the National Institute in Bethesda has published[52] some data on the disappearance of metachromasia following treatment with commercial testicular hyaluronidase and with amylase preparations Upon incubation with either fresh or boiled solutions of these materials the metachromasia of cartilage slices or of the basal membrane of arteries disappears The agent responsible for this change in metachromatic staining has been called a chondromucinase [52]

Gross Won t trypsin do the same thing?

Meyer Yes trypsin will do this in some tissues, but the enzyme of Lillie was heat stable to a prolonged boiling a treatment trypsin would not be expected to withstand Maybe there was a proteolytic enzyme in these enzyme preparations of Lillie

Gross What I am referring to is this just because the metachromasia disappears it doesn t mean that the agent producing the metachromasia has been destroyed It may be simply that the linkage to the tissue has been destroyed and the metachromatic material has been washed out

Meyer That is possible Another possibility is that these preparations contain a sulfatase However purified or crude hyaluronidases do not *in vitro* split sulfate from chondroitin sulfate

Japanese authors[53] showed that there are different kinds of sulfatases especially phenol sulfatases and glucosulfatases The phenol sulfatases are found in animal tissues and in plant material

Follis Taka diastase contains a sulfatase as is well known and it doesn t take out metachromasia

Meyer Taka diastase contains a phenol sulfatase That is different The phenol sulfatases do not attack sulfate esters of carbohydrates

Follis Well that is the only substrate that has been used isn t it?

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[52] Lillie R D Emmart E W and Laskey A M Chondromucinase from Bovine Testis and the Chondromucin of the Umbilical Cord *Arch Path* 52 363 368 (1951)

[53] Soda T *Sulfatases Methods of Enzyme Research* Leipzig (1941)

Meyer Phenolic sulfatases are widely distributed in bacteria, for example In tissue, the presence of an aliphatic sulfatase has been claimed by Neuberg [84] but the evidence was very poor and the experiments were not reproducible even in his own laboratory We are at present engaged in a study of the hydrolysis of the sulfate groups from chondroitin sulfates but thus far have failed to find in the body evidence for the presence of such a sulfatase

Follis There are phenol sulfatases in tissues?

Meyer Yes, they have been found in tissues, in plants, and in bacteria but there are very few sources of aliphatic sulfatases

Hastings Why is Dr Follis so anxious to establish that these phenol sulfatases exist in tissues?

Follis We have studied cartilage, using phenol sulfatase as a substrate

Meyer There are quite a few papers on the subject Recently, in London I heard of the hydrolysis of steroid sulfates The interesting point was the specificity of the enzyme which I believe were of bacterial origin An enzyme did hydrolyze the estrogenic sulfate hormones which have an aromatic OH while the enzyme did not split steroid sulfate with a hydroaromatic sulfate The specificity goes that far Soda[83] has shown that the sulfatases which hydrolyze phenol sulfates are distinct from those hydrolyzing glucose sulfate and chondroitin sulfate and Neuberg[84] has shown the same in some bacteria

The Presence of Mucoproteins in Tissues

I would like to say a word more about the fundamental and interesting work on fibrillogenesis of Dr Highberger and Dr Gross I would like to point out that there is no evidence that these mucoprotein fractions, which induce the 640 Angstrom orientation, contain uronic acid, or are derived from connective tissue

Fremont Smith You say there is no evidence?

Meyer There is no evidence This is a very fundamental point How can we explain the observations that substances which apparently do not occur in connective tissue induce collagen orientation, while substances which are known to occur in connective tissue either do not cause any orientation or only the 2200 artificial spacing, as Dr Gross called it?

Gross We think the reason perhaps, that there is no evidence for the

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[84]Neuberg C and Cahill W M The Enzymatic Splitting of Chondroitin Sulfuric Acid *Enzymologia* 1 22 38 (1936)

presence of this so called serum mucoprotein in the tissues is that nobody has yet tried to extract it and chemically characterize it Actually we have extracted from connective tissue a material which will produce the long spacing' fibril and collagen, in a manner similar to that of serum mucoprotein, and I think we have been able to demonstrate that probably there is such material in the tissues Whether or not it is the same as the serum mucoprotein of Weimer, Mehl and Winzler,[85] or of Schmid,[86] we don't know but Dr E J Cohn recently has said that this particular acid serum mucoprotein or glycoprotein that we have been working on is dialyzable through the blood vessel wall He did not give the evidence for it, but just made that as a bald statement If it is true that this small molecule does go through the blood vessel wall, one would expect to find it in the tissue

Meyer After isolation

Gross But there is no evidence as yet no experimental evidence, that it is present in the tissues However there is also none against it being present

Conference Discussion

Armstrong Are there other comments?

Follis Dr Meyer, would you repeat what you said about the combination between the polysaccharide and the protein?

Meyer Well, I don't know what I did say exactly What I attempted to bring out was the question of whether or not the polysaccharides of the supporting tissues are firmly bound to proteins, and whether or not the proteins present in such combination are specific proteins of the connective tissue and of the cartilage The firmness of the bonds or the types of bonds between carbohydrate and protein apparently differ considerably Some we consider as entirely polar bonds, that is, salt like bonds In cartilage we are not absolutely certain that we are dealing with polar bonds, while in the chondroitin sulfates of skin heart valves and tendon we definitely are not dealing only with polar bonds between chondroitin sulfate and protein Even after extraction with half saturated lime water, the 'Kalkmilch' of the old German histological literature the bonds between chondroitin sulfate and protein are not broken and sulfated mucoids prepared from such extracts will show an electrophoretic mobility of about 8, against a mobility

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[85]Weimer H E Mehl J W and Winzler R J Studies on the Mucoproteins of Human Plasma V Isolation and Characterization of a Homogenous Mucoprotein *J Biol Chem* 185 561 568 (August 1950)

[86]Schmid K Preparation and Properties of an Acid Glycoprotein Prepared from Human Plasma *J Am Chem Soc* 72 2816 (June 1950)

of about 11 for the isolated mucopolysaccharides under the same conditions But treatment of tendon or skin with 0 33N NaOH even at 0°, for a short time will rupture the bonds between protein and polysaccharide

In the neutral mucoids such as ovomucoid or the mucoproteins of Winzler treatment with boiling normal alkali will not break the bond or bonds between protein and carbohydrate Even digestion with trypsin will not lead to a peptide free carbohydrate

Follis One other question Dr Meyer When you started, you used the term 'ground substances,' which always waves a red flag at me What is your interpretation of 'ground substance'? The polysaccharide?

Meyer Oh no The definition is purely histological—that which is between the fibers or formed elements

Gross It is a perfectly good term

Meyer I think it is a good term This is the amorphous nonstructured substance or material which is between the fibers or in the cartilage

Follis Well is it collagen then?

Gross No, we are talking about matrix, and matrix is the fiber and the interfibrillary material or the ground substance

Meyer Well I would say that 'matrix' and 'ground substance' are synonymous I don t know, am I wrong?

Follis Would you comment on that, Dr Gross? What is your conception?

Gross Yes I think you could define the extracellular substance, being made up of the fibrous component and the interfibrillar component and the ground substance just by definition is the interfibrillar component That makes a pretty broad definition You can put anything in there

Follis Which of those two is collagen?

Gross Collagen is one of the fibrillar components

Hamilton That would be the nonamorphous component

Follis Is that a new definition of collagen?

Gross Collagen can be defined as a class of fibrous proteins characterized by a particular wide angle x ray diffraction pattern, and in the case of the known mammalian collagens, a rather characteristic amino acid composition

Follis Yes I know But to do something with it and get something out

must I put it under an electron microscope before I can say I've got collagen?

Gross No I think you can tell that there is collagen present

Follis How can I though? I mean that is the point that interests me

Meyer I think that if a material has a certain ratio of hydroxyproline to glycine you would call it collagen

Gross Maybe

Meyer With the introduction of procollagen the question is no longer a simple one

Hamilton I am raising the question that we have no definition of collagen

Gross No we think there are two definitions of collagen or two ways of characterizing collagen which we don't believe are completely unique but which are more unique than the histological appearance by the wide angle diffraction pattern and by the electron microscopic appearance of the fibrils Now maybe these definitions are too narrow but we think at the moment they are the best ones that we have

Fremont Smith A name for a substance is often looked at quite differently by different departments of science depending upon the particular technical tools or measuring devices used by each Would it not clarify the situation if instead of trying to decide which use of the word collagen or the term ground substance is correct we would specify exactly what is meant when the word or term is used Possibly this group might specify the two three four or five ways of defining collagen I don't believe there is any place in the literature where the meaning of the world collagen may have for different scientists has been clearly stated

Armstrong Gentlemen I don't want to stop the discussion but I am going to ask Dr Hamilton and Dr Meyer and Dr Gross to formulate definitions of matrix collagen and so forth upon which they can agree These definitions upon which there is unanimity of opinion will be inserted into the minutes

Fremont Smith Oh please let's not ask them to agree! Would it not be better to ask them to specify their disagreement so that we get several definitions of collagen?

Armstrong I think these gentlemen will come to an agreement Dr Fremont Smith

Fremont Smith I hope they won't!

Follis Dr Gross it is of interest that if Partridge[87] is right and there is only one protein in cartilage and that is collagen the fibers that one can get out of cartilage form the fibrillar material I should think the only conclusion would be that the interfibrillary material must be the chondroitin sulfuric acid and salts

Gross But actually Partridge did not present any definite data as to the nature of the protein

Follis He found a single protein

Gross What he found were three electrophoretic peaks One of them looked like chondroitin sulfate

Follis I have the article right here if anyone would like to see it

Meyer As far as I remember he got the third peak in the ascending limb only It was not present in the descending The same problem has been described with others of these highly polar polyelectrolytes Partridge himself is not very clear about his conclusions He says in one place that the third peak represents an artefact

Follis Nucleoproteins have been demonstrated of course

Gross What about the cytoplasmic proteins? Partridge doesn t find any evidence of these and yet they are there

Meyer You can only demonstrate them electrophoretically when they are present in a certain concentration

Copp I should think this is a very fundamental point and one would be reluctant to assume that there is only this one protein in osteoid matrix unless there were very convincing proof for it It seems a little difficult to assume that collagen of connective tissue which shows no affinity for metals or bone salts should be the same substance which in osteoid matrix shows a very marked affinity for bone salt and metals

Meyer It seems to me that if this is collagen then its solubility in neutral salt solution is quite different from the solubility of collagen anywhere else

Definitions of Collagen

Gross *Collagen* is a term embracing a class of fibrous proteins characterized by a particular wide angle x ray diffraction pattern many members of the class exhibiting a fiber axis period averaging 640 A The known

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[87] Partridge S M The Chemistry of Connective Tissues I The State of Combination of Chondroitin Sulphate in Cartilage *Biochem J* 43 387 397 (1948)

collagens of the higher animals are chemically characterized by a low content of aromatic amino acids and a high content of pyrolidine amino acids and glycine *Ground-substance* can be defined as the extracellular and interfibrillar amorphous component of all tissues

Hamilton *Collagen*—a term reserved for native fibrous proteins exhibiting specific spatial periodic wide angle x ray diffraction patterns and containing 6 to 7% of total N as hydroxyproline N, 10 to 12% as proline N, 1% as hydroxylysine N, and 25% as glycine N

Meyer The term *collagen* is ambiguous and is defined histologically (by microscopy in visible light and by submicroscopic technics) or chemically In the chemical definition, the term should be reserved for material from mammalian sources having a certain composition including the content of glycine, proline, and hydroxyproline, and hydrolyzed specifically by the enzyme, collagenase All fibers of similar composition and histological appearance might be called *collagenoid*

RADIOAUTOGRAPHIC STUDIES ON S^{35}-LABELED-SULFATE-SULFUR METABOLISM IN THE ARTICULAR CARTILAGE AND BONE OF SUCKLING RATS[88]

DOMINIC D DZIEWIATKOWSKI

From the Hospital of the Rockefeller Institute for Medical Research New York N Y

Armstrong I should like now, to ask Dr Dziewiatkowski to tell us something about the rate of turnover of the components of chondroitin sulfuric acid

Dziewiatkowski On the basis of some studies on the role of alkaline phosphatase in osteogenesis Siffert[89] advanced the opinion that 'there is *evidence to suggest that cartilage matrix is utilized in the formation of bone* matrix About two years ago Rubin and Howard[90] suggested that chondroitin sulfate plays a specific and important role in calcification I believe that the results of our studies on the metabolism of sulfate sulfur, tagged with sulfur[35] in the bone and cartilage of suckling rats emphasize the probable correctness of these opinions

Methods

In every instance but one S^{35} labeled sodium sulfate was injected intraperi ... ned to the mothers and ... The ... tibiae ... from ... The ... pH of this solution as determined with a glass electrode was ... other humerus tibia and femur from each animal were fixed in a 37% solution of formaldehyde saturated with barium hydroxide This solution was prepared 24 hours before use kept in the meantime at 25° C and fixation was allowed to take place for 48 hours at this temperature Under these conditions consistent results were obtained

The bones were imbedded in paraffin without decalcification cut at 7μ and the sections transferred to microscope slides coated with egg albumin The paraffin was removed with xylol and the mounted sections were covered in a dark room

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[88] The sulfur[35] used in these investigations was supplied by the Oak Ridge National Laboratories on allocation from the United States Atomic Energy Commission

[89] Siffert R S The Role of Alkaline Phosphatase in Osteogenesis *J Exper Med* 93 415 426 (1951)

[90] Rubin P S and Howard J E Histochemical Studies on the Role of Acid Mucopolysaccharides in Calcifiability and Calcification *Trans Macy Conference on Metabolic Interrelations* 2 155 166 (1950)

with rubber bands and were wrapped individually in black paper. The wrapped 'sandwiches' were stored in the dark at 0° C until by trial it was found that exposure was adequate. The sections were subsequently stained with toluidine blue 0.1% in 30% ethanol.

In some instances sections of humeri were floated onto the emulsion of lantern slide plates according to the directions of Evans.[8] In all cases the emulsions were developed, whether film or lantern slides, in the same fashion. If tissue from uninjected animals was used, no image was developed on the emulsions.

Results After Fixation with Acidic Formaldehyde

For the moment we will consider only the upper portion of Figure 19 a to 4a and 1b to 4b, which shows the reproductions of radioautographs b) and the corresponding stained sections (a) of humeri which were emoved from animals sacrificed 15, 30, 60 and 120 minutes after the dministration of the labeled sodium sulfate. These radioautographs are of humeri which were fixed in the acidic solution of formaldehyde. The ulfur^{35} is indicated as present throughout the epiphyses of the humeri but he highest concentration is at the epiphyseal diaphyseal junction. There is essentially no change in the pattern of deposition from the 15th minute to he 2nd hour, except for intensification.

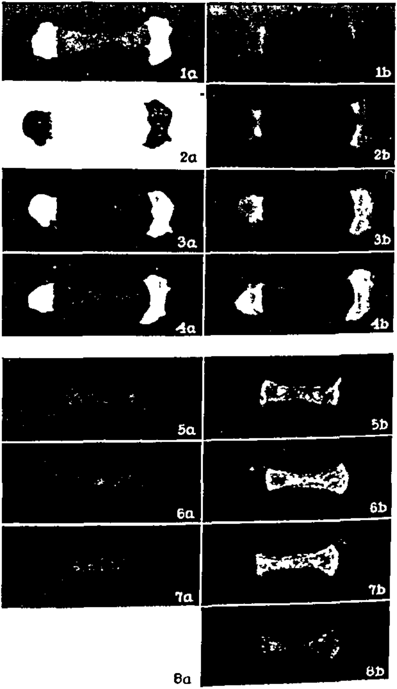
In Figure 20 are reproduced radioautographs (b) and the corresponding tained sections (a) of humeri removed 2, 4, 6, 10, 24, and 48 hours after ntraperitoneal administration of S^{35} labeled sodium sulfate. The sections were from humeri fixed in an acidic solution of formaldehyde. Again rom this series of radioautographs, one has evidence that after 2 hours ulfur^{35} is present throughout the entire epiphysis, but that it is more ighly concentrated at the junction with the diaphysis. The pattern of leposition does not change markedly up to the 24th hour except for in ensification. After the 24th hour, there seems to be a decrease in the ulfur^{35} concentration of the cartilage immediately adjacent to the diaphy is (14b). This is brought out more convincingly, I believe, in Figure 21.

In Figure 21 are reproduced the radioautographs (b) and the corre ponding stained sections (a) of humeri removed 24, 48, 96, 216, and 290 ours after labeled sodium sulfate administration. One can see that there s a better fusion of the two zones, one into the other by the 96th hour as

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[7] Eli Lilly and Co., Indianapolis, powder paper (Glassine) was used. The sections were ot covered by this paper.

[8] Evans, T. C. Radioautographs in Which the Tissue Is Mounted Directly on the Photographic Plate, *Proc. Soc. Exper. Biol. & Med.* 64: 313-315 (1947).


1a
1b
2a
2b
3a
3b
4a
4b
5a
5b
6a
6b
7a
7b
8a
8b

though a further distribution of the isotope throughout the epiphyseal cartilage were taking place One can certainly see a nearly even distribution of the sulfur[35] in the epiphyseal cartilage by the 216th and 290th hours In addition one can see that as centers of ossification develop in the epiphyses, there is a decrease in the concentration of some sulfur[35] bearing component here

Follis Is there a reaction in the shaft?

Dziewiatkowski Yes, there is a reaction in the shaft You can see the shaft outlined faintly and that possibly—this is just a speculation now—is an indication that there is some chondroitin sulfate or something that behaves like chondroitin sulfate in the shaft

Follis You have removed the marrow?

Dziewiatkowski No the marrow was not removed The bones were not decalcified The whole bone was fixed and sectioned

Sobel How do you know that the sulfate is attached to the chondroitin?

Dziewiatkowski This opinion is the result of experiments previously

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Fig 19 Sections and Radioautographs of Humeri of Rats Exposed to S^{35} Labeled Sodium Sulfate for 15 to 120 Minutes

METHOD OF PREPARATION The humeri were removed at various intervals of time (as indicated below) after the intraperitoneal administration of about 3 μc of sulfur[35] in the form of sodium sulfate to 7-day old litter mates fixed (as indicated below) sectioned, utilized for radioautographs (as indicated below) and stained with toluidine blue The reproductions of the sections were made by using the histological sections as negatives The radioautographs were produced from the sections by exposing the sections to Kodak contrast process ortho film for intervals of time (as indicated below) The films were used as negatives in making the enlargements and reproductions of the autographs In these reproductions therefore the lighter areas correspond to the darker areas in the original radioautographs

Sections 1a 2a 3a and 4a from humeri which were removed at 15 30 60 and 120 minutes respectively, were fixed in a solution of formaldehyde and were utilized in producing *Radioautographs 1b 2b 3b and 4b* respectively by exposing the film for 1 week *Sections 5a 6a 7a and 8a* from humeri which were removed at 15 30 60 and 120 minutes respectively, from the same rats as the humeri used to obtain sections 1a 2a 3a and 4a respectively were fixed in a solution of formaldehyde saturated with barium hydroxide and were utilized in producing *Radioautographs 5b 6b 7b and 8b* respectively by exposing the film for 3 weeks

RESULTS In *Sections 1a, 2a 3a and 4a* the epiphyseal cartilage is heavily stained In *Radioautographs 1b 2b 3b and 4b* a progressive deposition of sulfur[35] in a characteristic pattern in the epiphyseal cartilage can be seen while the shaft is only faintly outlined. In *Sections 5a 6a 7a and 8a* the epiphyseal cartilage is only faintly stained In *Radioautographs 5b 6b 7b and 8b* a deposition of the sulfur[35] in the bone shaft newly formed bone and bone marrow is apparent and it can be seen that a relatively large amount of the isotope accumulated transitorily in the bone marrow while the epiphyseal cartilage gave only a faint reaction

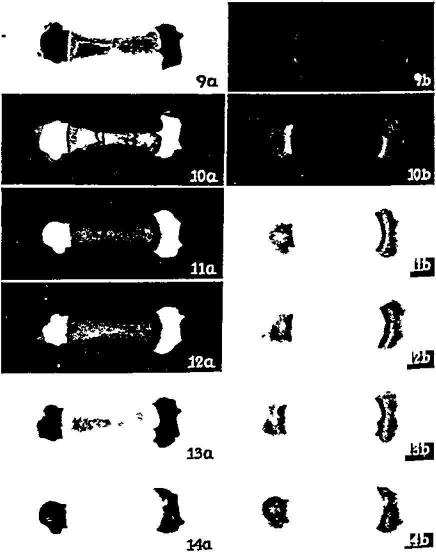


Fig 20 Sections and Radioautographs of Humeri of Rats Exp ed to S35 Labeled Sodium Sulfate for 2 to

METHOD OF that the rat ntraperiton rg f rmald

v The humeri w bout 125 μc of n and the sect

as descr bed n F g 19 except e form of sod um sulfate by only in the solution conta n

13a a d 14 vely wer a tograp 1 week

ere removed at 2 4 formaldehyde an l and 14b respec

done[9] in which chondroitin sulfate was isolated and found on repeated reprecipitation to undergo no change in the specific activity

Meyer You isolated the chondroitin sulfate from this epiphysis?

Dziewiatkowski No from the knee joint cartilage of rats of this age The chondroitin sulfate in which the sulfur was tagged with $sulfur^{35}$ was isolated from 9 day old rats that were given labeled sodium sulfate intraperitoneally 48 hours previously

Meyer Do you have any quantitative data of the amount of the total radioactive sulfate in the chondroitin sulfate?

Dziewiatkowski No I do not

Neuman As I remember that paper you recovered the bulk of the radioactivity in the crude chondroitin preparation

Dziewiatkowski That s right in the paper you have in mind[94] about 90 per cent of the $sulfur^{35}$ was shown to be present in the crude chondroitin sulfate preparation In answering Dr Meyer I thought that the data on the specific activity of the sulfur in chondroitin sulfate as one might isolate it without carrier were wanted We have not determined this

The Loci in Tissue of the Preferential Deposition of Radiosulfur

Why there is this preferential deposition of $sulfur^{35}$ at the junction of the epiphysis with the diaphysis it is impossible to say It may be due to a difference in composition or metabolic activity

Gross Are there more cells in that area?

Follis Is this the place of growth of the cartilage?

Dziewiatkowski That s right if you consider increase in size of cartilage cells as growth

[93]Dziewiatkowski D D Isolation of Chondroitin Sulfate S^{35} from Articular Cartilage of Rats *J Biol Chem* 189 187 190 (1951)

[94]Dziewiatkowski D D Benesch R E and Benesch R On the Possible Utilization of Sulfate Sulfur by the Suckling Rat for the Synthesis of Chondroitin Sulfate as Indicated by the Use of Radioactive Sulfur *J Biol Chem* 178 931 938 (1949)

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RESULTS In *Sections 9a 10a 11a 12a 13a and 14a* the epiphyseal cartilage is heavily stained In *Radioautographs 9b 10b 11b 12b 13b and 14b* a progressive deposition of the $sulfur^{35}$ in a characteristic pattern in the epiphyseal cartilage can be seen up to about the 24th hour after injection (Radioautograph 13b) A fading of the zone of highest concentration at the epiphyseal diaphyseal junction is seen to have occurred by the 48th hour after injection (Radioautograph 14b)

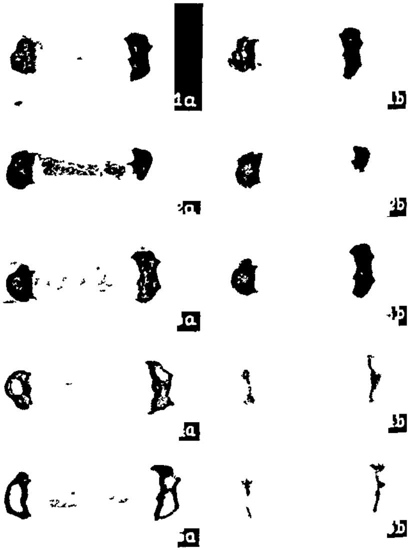


Fig 21 Sections and Radioautographs of Humeri of Rats Exposed to S^{35} Labeled Sodium Sulfate for 24 to 290 Hours

… in Fig. 1) except
ly 10 µc.) by
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…moved at 24 48 96 216
formaldehyde and were
respectively by exposing

Copp I would expect that the very active formation of new cartilage in that area would account for the relatively high specific uptake

Dziewiatkowski There is a hypertrophy of the cartilage cells as I understand it, and an extension of the matrix, where these cartilage cells are found

Follis Isn't the whole epiphysis radioactive?

Dziewiatkowski Yes, it is

Armstrong The whole epiphyseal cartilage appears to be uniformly filled with radioactive material

Dziewiatkowski Well, it may be difficult to see that there are differences in the concentration of sulfur[35] in different areas of the epiphyseal cartilage of the humeri removed after 24 to 48 hours The differences can be made out without difficulty in the original radioautographs In these reproductions too differences can be made out I think you can see it more convincingly presented in Figure 20

Neuman This gets back to the earlier question If radiosulfate—and by that I mean ionic sulfate—does not undergo exchange with preformed chondroitin sulfate, then these results are a definite indication of the synthesis probably extracellular, of new chondroitin sulfate from inorganic sulfate

Armstrong Exactly

Dziewiatkowski That's right

Johnson Did you include this zone in the cartilage section you used to isolate chemically the chondroitin sulfate preparation which had 90 per cent of the activity?

Dziewiatkowski Yes, indeed

Johnson And the area of new forming bone?

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[Modified and reproduced by permission from Dziewiatkowski D Radioautographic Visualization of Sulfur[35] Disposition in the Articular Cartilage and Bone of Suckling Rats Following Injection of Labeled Sodium Sulfate *J Exp Med* **93** 451 458 (1951)]

Dziewiatkowski Yes In these young rats you can separate the epiphyses from the bone shaft very easily

Johnson When you make the separation you know for sure which side or which fragment the dense line goes with?

Dziewiatkowski Do you mean in the chemical separation?

Johnson No when you physically separate the epiphysis from the shaft do you know for certain with which of those two pieces the dense line goes?

Dziewiatkowski I m sorry but I don t understand what you are driving at May I restate what we did and perhaps thereby answer your question For the isolation of the chondroitin sulfate we took the knee joints of 8 to 9 day old rats The knee joints were easily and cleanly separated from the shaft

Johnson My point is though in separating the joint from the shaft do you know for certain whether this dense line goes with the joint or does it stay with the shaft?

Copp I would think the chemical results showed that

Dziewiatkowski Yes I think it goes with the joint The joint separates from the shaft cleanly

Follis Could not this dense area be sulfate which is being deposited with the other inorganic materials and not as chondroitin sulfate?

McConnell That is the question I was about to ask because it has been adequately demonstrated that some of these calcium phosphates the type of which teeth and bones are composed are subject to isomorphic substitutions of exactly this nature You can have a silicate sulfate with the same crystal structure as apatite

Dziewiatkowski I hardly think this is inorganic sulfate as such One would expect inorganic sulfate to be removed in the process of fixation in the acidic formaldehyde

Johnson An alternative interpretation has occurred to me and this was the reason that I was asking the question as to the precise location of the dense line In the resorption and breakdown of the cartilage matrix that is going on in the zone of cartilage *maturation* is it possible that some of those breakdown products of the polysaccharide series may become excessively sulfated like the heparin substances? The dense line may have nothing to do with the chondroitin sulfate but instead represent excess sulfate picked up by the partially split products of the early stage of the

breakdown of chondroitin sulfate Therefore the proof that this dense line is actually with the joint fragment at the time of physical separation is necessary in order to know what is going on there

McConnell As an answer to my question then do you imply that these are decalcified tissues? Is that right?

Follis He said they were not decalcified

McConnell Well then the sulfate cannot go into the apatite structure I believe

Dziewiatkowski Let me put it this way there was no intention of markedly decalcifying the tissues One would not expect extensive decalcification in the acidic formaldehyde of pH 3 8 to 3 9

Neuman The bulk of the calcium phosphate is in those areas where the sulfate isn t found

Copp I would think that is perfectly clear

Follis I don t think that it is clear

Copp In these young animals the separated epiphysis does not contain bony material but is cartilage and the area which shows such an intense radioactivity I would say corresponds to the area of proliferating cartilage and not to the calcifying area below it

Dziewiatkowski Proliferating and also mature cartilage cells

Copp As you go farther down toward the zone of mature cartilage radioactive sulfur is no longer present and you apparently do not get sulfate uptake

Now when you separate the epiphysis the piece that comes off in your hand has a proliferating cartilage and the piece that is left behind on the shaft is calcified or contains the region in which calcification is taking place

McConnell Isn t that the area in which the calcification would take place if the calcification took place?

Dziewiatkowski Yes it is the area in which calcification will take place in the near future

Copp No not that area but the area immediately below it With radiocalcium the latter would show very high activity while the piece which comes off with the epiphysis and which is cartilaginous shows in radioautographs no calcification and no radiocalcium uptake I think there is a clearcut separation here between cartilage production and calcification

Johnson The problem that bothers us all is that we don't see the histology, so we don't know exactly what you take off for your joint, we don't know exactly what zones of cartilage are included

Dziewiatkowski I have an illustration on which I can indicate that May I proceed then?

Armstrong Please do

Dziewiatkowski It is possible to show that this area of intense deposition of sulfur[35] is associated with the more mature cartilage cells For example if S^{35} labeled sodium sulfate is injected into a 15 day old rat (Figure 22), the radioautographs of the humeri removed 24 hours later show two zones of relatively high sulfur[35] concentration one, an area at or near the junction of the cartilage and the diaphysis, and the other an area surrounding the fairly well developed center of ossification in the epiphysis A photomicrograph (Figure 23) of a portion of a section from a humerus of a rat at this age shows that there are mature cartilage cells at the junction of the epiphysis and the diaphysis Around the ossification center in the epiphysis, also, there are mature cartilage cells

Follis Where do you think you break that off?

Dziewiatkowski In the removal of knee joints for analysis we endeavored to break off the cartilage from the shaft as cleanly as possible so as to retain all of the cartilage, but not to include any of the shaft The cartilage above a line drawn through the most mature cartilage cells those in the process of removal was the cartilage we tried to obtain for analysis

Follis Then you have inorganic material there which could contain sulfate

Dziewiatkowski Yes, but I believe I can demonstrate that the highest sulfur[35] concentration is not in the region of the most mature the disintegrating cartilage cells The highest sulfur[35] concentration is associated with the cartilage cells which are a step or two removed from the stage at which they give way to bone

Follis Where there is more matrix?

Dziewiatkowski Yes, where there is more matrix

Follis And there is more matrix there than there is up above?

McLean Let's clarify the morphology before you go ahead Would you like to do that?

Dziewiatkowski Yes, please do

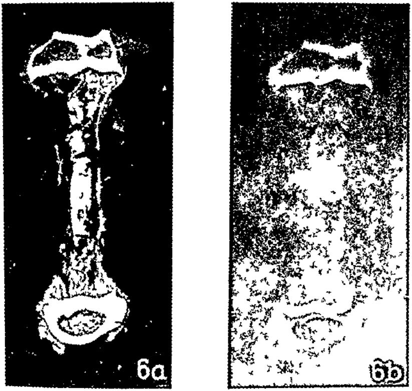


Fig 22 Sections and Radioautographs of Humerus of a Rat Exposed to S^{35} Labeled Sodium Sulfate for 24 Hours

METHOD OF PREPARATION The humerus was prepared as described in Fig [illegible] that a 15 day old rat was given 4 mg of labeled sodium sulfate (approximately 165 μc) by intraperitoneal administration and the section was fixed [illegible] solution containing formaldehyde alone From *Section 6a Radioautograph* [illegible] prepared by exposing the film for 4 weeks

RESULTS In *Section* [illegible] the epiphyseal cartilage is heavily stained [illegible] *graph 6b* the sulfur[35] can be seen to be deposited in two zones [illegible] relatively high concentration one at or near the junction of the cartilage and the [illegible] and the other surrounding the fairly well developed center [illegible] ossification in the [illegible]

[Modified and reproduced by permission from Dziewiatkowski D Radioautographic Visualization of Sulfur[35] Disposition in the Articular Cartilage and Bone of Suckling Rats Following Injections of Labeled Sodium Sulfate *J Exp Med* **93** 451 458 (1951)]



Fig 23 A Photomicrograph Radioautograph of Epiphyseal Cartilage of the Humerus of a Rat Exposed to S^{35} Labeled Sodium Sulfate for 24 Hours

METHOD OF PRE
the diaphysis of
Radioautograph I
a Kodak contrast
developed with th
micrograph was made

1

McLean Please refer to Dr Dziewiatkowski's sections 1a to 5a (Figure 19) Note that there are two kinds of cartilage in these sections In the younger animals there is a cartilage model of what will eventually become the epiphysis of the bone, and within a few days it is invaded partly removed and replaced with primitive bone marrow The other cartilage that showing the highest concentration of S^{35} is that destined to become the growth zone and it is, as Dr Dziewiatkowski has said actively proliferating None of the cartilage model is calcified at all and there will be no deposition of mineral in it until the cartilage is removed and replaced by bone Under higher power (Figure 22) the different kinds of cartilage are clearly seen First there is hyaline cartilage which never will calcify but which in this section is being replaced by bone Then there is a zone of proliferating cartilage with the cells arranged in rows each with a r w mother cell Finally the cells in the rows mature or hypertrophy and they are removed in the process of ossification Only in the matrix in contact with these hypertrophic cells is there deposition of mineral salt It is clear then from Dr Dziewiatkowski's radioautograms that the intensity of the radiation from S^{35} deposited in the hyaline cartilage is not correlated with any deposition of bone mineral in that tissue

Dziewiatkowski I may have said 'undecalcified What I meant to say was that the bones were not intentionally decalcified as is usually done by the use of dilute nitric acid or other dilute acid The bones were fixed in acidic formaldehyde the pH of which was 3 8 to 3 9 between and then carried through dehydrating alcohols and imbedded in paraffin Some decalcification might have occurred I think it did but it probably was very slight

In Figure 22 which is the reproduction of a radioautograph of a section from a humerus removed 24 hours after administration of S^{35} labeled sodium sulfate to a 15 day old rat you can see that two zones of relatively high S^{35} concentration are indicated One of these zones is again seen at the junction of the epiphysis with the diaphysis The second zone is present as a ring around the ossification center in the epiphysis

McLean That is where the identical process is going on

Dziewiatkowski Yes it is further possible to say that the ultur[35] n

---

RESULTS The number of grams of reduced silver is not as great in the areas ... by the most mature cartilage cells such as those seen in the bottom ... a it is in the areas covered by slightly younger cell

[Modified and reproduced by permission from Dziewiatkowski D Radioautographic Visualization of Sulfur[35] Disposition in the Articular Cartilage and Bone of Suckling Rats Following Injection of Labeled Sodium Sulfate *J Exp Med* 93 451 4 9 (1951)]

centration is not as great in the region of the most mature cartilage cells as it is in the region of only slightly younger cells Evidence for this was found in radioautographs of humeri when the radioautographs were prepared according to the method of Evans[92] A photomicrograph of part of such a radioautograph is presented in Figure 23 One sees in such magnified radioautographs that the concentration of silver grains is not as great in the immediate vicinity of the most mature cartilage cells as it is in the region just above them

Results After Fixation with Formaldehyde Saturated with Barium Hydroxide

Radioautographs of humeri fixed in a solution of formaldehyde saturated with barium hydroxide do not resemble those of humeri fixed in a solution of formaldehyde alone Representative radioautographs and the corresponding stained sections of humeri removed from rats 15 minutes to 120 minutes after S^{35} labeled sodium sulfate administration are given as 5a to 8b Figure 19 By the end of 15 minutes, it is seen in 5b that the bone shaft and bone marrow have already acquired a large fraction of the tracer isotope which is to be deposited therein up to the second hour From these radioautographs it also appears that $sulfur^{35}$ is transitorily but to a relatively large extent deposited in or on some component(s) of the bone marrow up to at least 30 minutes (Figure 19 6b) By the end of 60 minutes (Figure 19 7b) the discrete localization of the radioisotope is less apparent and after two hours is almost indiscernible

It is of interest to note that not only the radioautographs but the staining characteristics of the humeri are dependent upon the method of fixation The epiphyses of humeri fixed in a solution of formaldehyde take up toluidine blue and are stained a deep purple On the other hand the epiphyses of humeri fixed in a solution of formaldehyde saturated with barium hydroxide take up the dye to only a very slight extent in the same period of time and are stained a faint blue It has been claimed that toluidine blue stains chondroitin sulfate characteristically and specifically[90 95 96] and it has been shown that the barium salt of chondroitin sulfate is very soluble in water[97] Therefore the marked loss of affinity for toluidine by the cartilage of the bones fixed in a solution of formaldehyde

[95]Sylven B Occurrence of High Molecular Sulfate Esters in Granulation Tissue and in Epithelial Regeneration *Acta chir scandinav* 86 Suppl 66 1 151 (1941)

[96]Sylven B Ester Sulphuric Acids of High Molecular Weight and Mast Cells in Mesenchymal Tumors Histochemical Studies on Tumorous Growth *Acta rad ol* Suppl 59 1 99 (1945)

[97]Bray H G Gregory J E and Stacey M Chemistry of Tissues Chondroitin from Cartilage *Biochem J* 38 142 146 (1944)

saturated with barium hydroxide can be due to the extraction of chondroitin sulfate during fixation

In Figure 24 15a through 22b, are shown the stained sections (a) and the corresponding radioautographs (b) of humeri fixed in a solution of formaldehyde saturated with barium hydroxide These humeri were removed from litter mates sacrificed 2 hours to 96 hours after administration of labeled sulfate It can be seen that sulfur^{35} is present in some component(s) of the bone marrow even at 96 hours after administration (Figure 24 22b) In the original radioautographs it appeared that between the 2nd and 24th hours the concentration of the sulfur^{35} containing component(s) of the bone marrow increased and thereafter slowly decreased

One sees also that in the bone shaft the S^{35} labeled component(s) is rather diffusely deposited from the 2nd to the 10th hour (Figure 24 15b to 18b) By the 24th hour and thereafter (Figure 24 19b to 22b) the shaft is more sharply outlined

In all instances radioautographs of humeri fixed in a solution of formaldehyde saturated with barium hydroxide indicate a relatively high concentration of labed component(s) in newly calcified bone at the ends of the diaphysis Here the tracer is deposited diffusely up to the 24th hour Thereafter detail of structure is sharpened and intensified

In Figure 24 21b and 22b one sees also that some S^{35} labeled component(s) is present in epiphyseal ossification centers As the centers develop an increasing amount of the tracer isotope is incorporated into some component(s)

The Rate of Uptake and Decrease of Radiosulfur in Epiphyseal Cartilage

It is known that the number of developable silver halide grains of a photographic emulsion within certain limits is dependent upon the concentration of the radioactive source to which the emulsion is exposed[99] The number of silver grains present in a given area of a developed film is directly reflected in the optical density of that area It may be of interest to note that by determining the optical density of radioautographs produced by the epiphyseal cartilage fixed in formaldehyde it is possible to quantitate roughly the relative concentration of sulfur^{35} in the epiphyseal cartilage from one time interval to another To this end a background reading of each film was made with a Weston Photographic Analyzer Model 877 This background reading was then subtracted from half the sum of the average of three density readings taken in the area of highest concentra

[99] Cobb J and Solomon A K The Detection of Beta Radiation by Photographic Film *Rev Sci Instruments* 19 441-447 (1948)

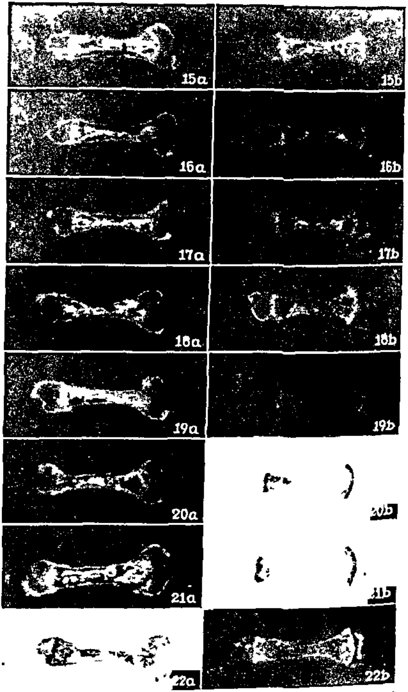


Fig 24 Sections and Radioautographs of Humeri of Rats Exposed to S^{35} Labeled Sodium *Sulfate for 2 to 96* Hours

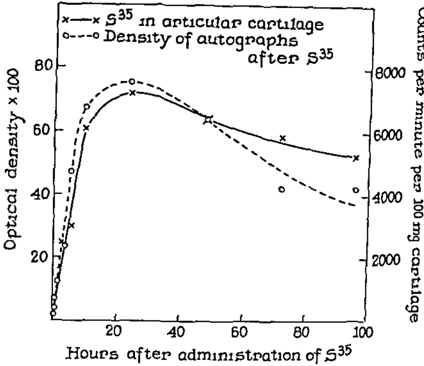


Fig 25 The Relationship with Time of the Intensity of the Radio autographic Reaction of the Epiphyseal Cartilage of 7 Day Old Rats after the Intraperitoneal Administration of S^{35} Labeled Sodium Sulfate

The intensity of the react on was measured as optical density with a Weston Photograph c Analyzer Model 877 The value at any time interval is the average obtained from all of the rad oautograpl s of humeri and tibia femur comb nations that were removed at that time interval and fixed in the solution of formaldehyde For comparison there are included the previously reported data of Dziewiatkowski[93] on the accumulation of sulfur[35] in the knee joint cartilage of 7 day old rats after intraperitoneal injection of labeled sod um sulfate

saturated with barium hydroxide To a large extent those components which are insoluble in a) appear to be soluble in b) and *vice versa*

It is suggested that the *formaldehyde insoluble sulfur*[35] is for the most part present as chondroitin sulfate and may, therefore be an index to the *rate of chondroitin sulfate turnover in the articular cartilage* of the suckling rat This suggestion is based on the following it has been found that sulfur[35] when given as sodium sulfate is incorporated into chondroitin *sulfate of the articular cartilage in the suckling rat*[93] The major portion of the sulfur[35] which is retained in the articular cartilage is probably retained therein as chondroitin sulfate[94] The loci in tissue where the formaldehyde insoluble sulfur[35] *is demonstrable* by radioautography are the loci which show metachromatic staining with toluidine blue

If the formaldehyde insoluble sulfur[83] represents chondroitin sulfate its deposition indicates that the synthesis of chondroitin sulfate takes place throughout the entire epiphyseal cartilage The rate of chondroitin sulfate turnover however is not the same in all parts of the cartilage The rate of turnover is highest in the epiphyseal region adjacent to the diaphysis and is accelerated in the cartilage which immediately surrounds developing centers of secondary ossification The inference which may be drawn from such an explanation is that calcification is associated with an accelerated synthesis and removal of chondroitin sulfate in the adjacent cartilage

Sulfur[35] which is insoluble in a solution of formaldehyde saturated with barium hydroxide is probably inorganic sulfate sulfur and sulfur in any form insoluble as a barium salt at a slightly alkaline pH Sulfur so characterized is demonstrable in increasing concentration in developing centers of secondary ossification In the diaphysis adjacent to the epiphysis where new bone is being laid down there is also a progressive increase in this form of sulfur The sulfur containing component(s) laid down in these areas of calcification may obtain the sulfur in part from the chondroitin sulfate of the adjacent cartilage This would be in line with the opinion expressed by Siffert[89] as regards utilization of cartilage matrix in the formation of bone matrix and the opinion of Rubin and Howard[90] that chondroitin sulfate plays an important role in calcification

Sulfur in components which are insoluble as barium salts is present in the bone shaft and once accumulated is demonstrable therein for at least 12 days

The radioautographs of bone marrow may be interpreted as indicating the presence of sulfur almost entirely in forms which are insoluble as barium salts The rapid and relatively large transitory deposition of some of the tagged sulfate sulfur in discrete foci suggests the following sulfur in the form of inorganic sulfate sulfur enters the bone marrow rapidly but is also rapidly removed or incorporated into a more complex component From the more complex component the sulfur is then removed slowly

From all of the observations presented one can visualize a dynamic disposition of sulfate sulfur in the articular cartilage and leg bones of the suckling rat from age 7 days to 19 days after birth

Conference Discussion

Armstrong What does the barium hydroxide do? Does it precipitate the inorganic sulfate present in the extracellular fluids of the tissue?

Dziewiatkowski Yes one would expect it to do that The reasons that the tissues were fixed in acidic formaldehyde and in formaldehyde saturated

Dziewiatkowski is the content calculated to the total amount of bone or is it calculated in relation to the non collagenic content? The total amount doesn't mean much It means something only if you calculate it with relation to the non collagenic content

Follis As I recall it he did cortex without any marrow elements and did it on a weight for weight basis

Johnson Well then it might not mean very much

Hastings I am sure he got results on materials like ear cartilage or something of that sort compared with noncalcified calcifiable cartilage That is one of the points of it but I can't remember the details well enough to say

Armstrong It would seem to me to be of interest to determine whether or not the sulfuric acid radical of chondroitin sulfuric acid freely exchanges with inorganic sulfate I personally would doubt that it does but the facts could very easily be demonstrated by fairly simple experiments Dr Follis has expressed surprise that cartilage could accumulate as rapidly as Dr Dziewiatkowski's results indicate

Follis I am surprised that chondroitin sulfate accumulates this rapidly

Armstrong Well if cartilage grows then the components also must accumulate I think we have plenty of examples in which the accumulation of protein is just as rapid a process as is apparently the case here

Neuman I think one of the startling things is that the chondroitin sulfate is being made from inorganic sulfate

Armstrong That point is not proved absolutely because there might be an intermediate in the synthesis

Neuman It is my impression that inorganic sulfate is not reduced to any extent in the body and that ordinarily most sulfur compounds are oxidized to sulfate This must be a direct esterification and it is hard to see how the energy relationships are satisfied unless it is a transesterification of some sort

Armstrong Well it is perfectly possible to conceive that intermediate sulfate compounds are prepared and handled in a process something like that of transmethylation

Neuman Some small amount of an active ester is formed and then that reacts with a sulfate acceptor?

Follis Of course there is a great deal there

Hendricks Why wasn't the answer that was given against simple turn over or exchange of inorganic sulfate satisfactory? The experiment was described in which the bone was placed in a system that, while it might not have kept the bone viable, yet at least kept the conditions there for exchange ideal It may not have kept all of the enzymes functional but it surely did the least possible damage to them, and there was no exchange under those conditions

Dziewiatkowski There was no significant uptake

Armstrong I think that such a conclusion can be drawn although the direct experiment has not been carried out

Hendricks You say there was no uptake?

Dziewiatkowski So little of the sulfate from the medium was fixed in the cartilage that it was not regarded as significant

Hendricks It looks to me as though that rules out all nonviable exchanges

Dziewiatkowski I hesitate to say that none could occur The conditions might not have been advantageous I am particularly hesitant because others have been able to demonstrate an uptake of sulfate by cartilage in similar media and we have not

Armstrong That is growth

Hendricks But that might simply mean that they maintained the synthesis of chondroitin sulfate, and you did not

Dziewiatkowski Yes, that may be so

Follis Did you keep the slice in contact with the radioactive sulfur as long as your experiments continued? A matter of days?

Dziewiatkowski Yes The tissue at the end of the experiment was rinsed repeatedly in isotonic saline The tissue and the medium were analyzed for sulfur^{35}

Johnson Why isn't it possible that the sulfate is just adsorbed on or associated with, some of the ancillary chemicals that are necessary for the synthesis of chondroitin sulfate and not going into the chondroitin sulfate molecule at all?

Armstrong Because he said the specific activity does not change

Dziewiatkowski We were able to isolate what is regarded as chondroitin sulfate, using the method of Bray, *et al* [97] On reprecipitation the specific activity of this material did not change

Hastings If it were phosphate we were talking about, you wouldn't be a bit surprised at that fast turnover

Hendricks No that's right

Copp Did you calculate how much of the chondroitin sulfuric acid in the epiphysis was actually synthesized during the experiment?

Dziewiatkowski No not in these experiments We did calculate the amount of chondroitin sulfate synthesized in other experiments,[100] in which varying amounts of sodium sulfate of the same specific activity were given to rats and their knee-joints were removed for analysis 24 hours later If I can recall that correctly, it was about 5 per cent of the dry weight and about 1 per cent of the wet weight, depending on how the calculation was made—whether on a dry or wet basis of newly laid down cartilage

Copp It seems very reasonable that, in an area of rapidly proliferating cartilage, so much new cartilage matrix should be synthesized

Dziewiatkowski I might add that it was disturbing to find the calculated values to be lower than any values reported in the literature

Sobel I think Dr Hastings' remarks, which in effect implies that chondroitin sulfate may undergo desulfurization similar to ATP, may be the real explanation of your phenomena As a matter of fact, according to Gamble's composition charts intracellular fluid has lots of inorganic sulfate to which no great attention has been paid Perhaps intracellular sulfate is an active metabolite in some of the metabolic processes and here Dr Dziewiatkowski has visually demonstrated one of these

Armstrong Do we really know that intracellular fluid contains inorganic sulfate, or do we employ a value which must be used in order to get a balance between positive and negative charges?

Hastings There may be some question whether that is inorganic sulfate but that is not the X The X is what is left over

Dziewiatkowski There is one other point I might mention in view of what Dr Sobel was saying The sulfur[35] which accumulates in the epiphyseal cartilage of the suckling rat after administration of labeled sulfate does not leave the cartilage quickly The peak concentration of the sulfur[35] in the cartilage is seen at about the 24th hour post injection Thereafter, up to the 164th hour, the decrease in concentration is slow

Neuman Another point which should be added here confirms the idea

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[100] Dziewiatkowski D D Effect of Thyroxine and Thiouracil on S^{35} Deposition in Articular Cartilage, *J Biol Chem* 189 717 727 (1951)

that there is no nonvital exchange of sulfate and that is the fact that sulfur[35], given as inorganic sulfate is excreted very rapidly The specific activity drops within a matter of hours precipitously If this were an on and off exchange we would expect it to leave the cartilage very rapidly which it does not

Meyer I think the question was asked by Dr Johnson as to whether one or additional groups were sulfated Your analysis ought to give you that answer The ratio of sulfate to uronic acid in the isolated chondroitin sulfate ought to give you that answer

Dziewiatkowski My data might give the answer to that question but I can t give it to you offhand

THE ION BINDING PROPERTIES OF CARTILAGE

WILLIAM F NEUMAN, EUGENE S BOYD and ISAAC FELDMAN

From the Division of Pharmacology and Toxicology Department of Radiation Biology School of Medicine and Dentistry University of Rochester Rochester N Y

Armstrong Dr Neuman will now discuss the physiologic role of chondroitin sulfuric acid in cartilage

Neuman I shall be brief for two reasons first the work I wish to report has just been published [102] Some of you may have seen it and some few of you may already have read it Second once a piece of work is accepted for publication I promptly forget all details concerning the research

This work had its point of departure in the discussions of the Conference of two years ago At that time there was a great deal of discussion of the importance of chondroitin sulfate As a result I came up with a perfectly wonderful idea that chondroitin sulfate by its nature must necessarily *bind cations in a fashion analogous to ion-exchange resins* I was horrified to find that I did not have a monopoly on this idea when a short time later Dr Stetten voiced this same suggestion [10] I kept quiet because I thought I would like to work on the problem Nothing came out in the way of a sequel to this from Dr Stetten s laboratory so when a graduate student Eugene Boyd and a physical chemist Isaac Feldman expressed an interest in this problem we began our studies

The Difference Between the Present Experiments and the Previous Studies

Earlier work on this problem was performed principally by two groups Freudenberg and Gyorgy[103] were *the originators of the idea of mineral* binding by cartilage They studied cartilage preparations that had a very low mineral content and concluded that calcium was bound first and phosphate only secondarily Subsequently Roche and Deltour[104] declared that

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[102] Boyd, E S and Neuman W F The *Surface Chemistry* of Bone V The Ion Binding Properties of Cartilage *J Biol Chem* **193** 243 251 (1951)

[10] Stetten D Jr *Trans Macy Conference on Metabolic Interrelations* 2 189 (1950)

[103] Freudenberg E and Gyorgy P *The Binding of Calcium* by Animal Tissue III *Biochem Z* **118** 50 54 (1921)

[104] Roche ... XII Role of Phosphatase in the Cal... Calcium XIII Role of Phosphata... Theory of the *Fixers of* Calcium

this was wrong They criticized Freudenberg and Gyorgy for using cartilage that had been obtained from areas relatively far from actively calcifying centers Accordingly Roche and Deltour selected as their preparations for study areas in the cartilage which contained a fair amount of preformed mineralization This point will be important in our subsequent discussion

The big difference between our work and that done previously is that we began with a preparation of *demineralized* cartilage Costal cartilage from veal was pulverized and then treated with Sequestrin which is I believe ethylene diamine tetra acetic acid In any event Sequestrin is a good chelating agent for calcium As calcium takes the form of a chelate complex phosphate goes into solution by itself Thus we were able to get a preparation of cartilage that was practically free of calcium and that contained only traces of phosphate which we presumed to be esterified

The Correlation Between the Cation Binding Capacity and the Sulfate Content of Cartilage

Our first experiments involved making a column containing the pulverized demineralized cartilage and passing various solutions of cations through this column until it became saturated with the particular cation under study Table I shows a series of analyses made with demineralized cartilage *(Preparation A)* We observed a definite stoichiometry one milliequivalent of cation was bound per gram of dry cartilage whether the cation were calcium barium or sodium Note that the sulfate content also analyzed as one milliequivalent per gram Thus the correlation between the cation binding capacity and the sulfate content is excellent

TABLE I

Cation Binding Capacity Sulfate and Phosphate Content of Demineralized Cartilage

Substance Analyzed	Ion Content m eq per gm Dry Cartilage — Cartilage			
	Preparation A		Preparation B	
	Number of Samples	Mean	Number of Samples	Mean
Ca	9	1 01 ± 0 08	8	0 62 ± 0 02
Ca (low pH)	4	1 01 ± 0 05	—	—
Ba	4	0 99 ± 0 05	—	—
Na	6	0 98 ± 0 03	—	—
Sulfate	5	1 02 ± 0 02	4	0 58 ± 0 01
Phosphate	1	0 025	—	—

We also treated this preparation of cartilage with potassium carbonate to remove part of the chondroitin sulfate *(Preparation B)* The resulting product as you see showed a lower binding capacity for cations There was still a one to one correspondence between the amount of cation bound and the sulfate content of the preparation

Actually the cation binding capacity in milliequivalents is exactly twice the number of ionized sulfate groups since the sulfate is present in the chondroitin as an ester We believe that the carboxyl group of glucuronic acid accounts for the other half of the capacity

Gross What was the pH of your solutions?

Neuman The pH was not set Unbuffered solutions were run through the column There was some drop in pH initially due to the exchange reaction but by the time equilibrium was reached all these solutions were near neutrality with the exception of one experiment with calcium chloride buffered at pH 4 6 As you can see the *capacity* is relatively independent of pH

Bartter What was the last point you made about the cation binding capacity in milliequivalents and the number of ionized sulfate groups not being one to one?

Neuman The amount of sulfate is calculated as one milliequivalent per gram but in the ester form one of the sulfate linkages is used up in forming the ester Therefore we would expect that the cation binding capacity resulting from sulfate would come out 0 5 milliequivalents per gram However taking into account also the cation binding capacity of the glucuronic acid carboxyl groups the total capacity should be one milliequivalent per gram which seems to be the case The glucuronic acid carboxyl group shows a fairly high degree of dissociation such that at the pH at which we were working it would be completely ionized and would bind cation

The Effect of Mineralization of Cartilage on Phosphate Absorption

In an attempt to get at the divergent results of Roche and Deltour[104] and of Freudenberg and Gyorgy [103] we tested our cartilage preparation for its ability to take up phosphate It did not When placed in a beaker with sodium phosphate solution or when phosphate solution was run through a column the cartilage did not take up phosphate ion We therefore did a series of experiments (Table II) in which we first treated with calcium chloride solution and then placed this calcium impregnated sample in phosphate buffer Under these circumstances we found that the cartilage took up phosphate and gave a calcium phosphate ratio of 2 to 1 approximating that of hydroxyapatite or bone This mineralized preparation was again

TABLE II

Results of Alternate Calcium and Phosphate Treatment of Demineralized Cartilage

Pretreatment	Solution Used	Duration of Experiment (hr)	Ca*	P*
None	$CaCl_2$	24	18	0.26
		30	18	—
$CaCl_2$	PO_4 buffer	20	16	6.9
		25	16	8.1
		40	16	8.0
$CaCl_2$ followed by phosphate buffer	$CaCl_2$	21	34	8.0
		23	33	7.7
		39	35	8.0
$CaCl_2$ PO_4 buffer followed by $CaCl_2$	PO_4 buffer	40	36	19.0
		50	53	20.0
		101	69	39.0

*Expressed as mg per gm of dry cartilage

placed in calcium chloride solution and again it took up calcium with no loss of phosphate. Now when this last preparation was again placed in phosphate buffer it took up phosphate in amounts much greater than expected. These last named results are of questionable quantitative value but they are of qualitative interest. It seems that once the cartilage has been mineralized it will absorb phosphate to a degree greater than can be accounted for by the apatite molecule. It must be remembered that we submerged the cartilage as did Roche in a very unphysiological concentration of phosphate (0.2 molar). I think therefore that Roche's results with mineralized cartilage can be explained on the basis of a phosphate absorption by the preformed mineral present in his preparation.

As a matter of fact if one examines such a sample of cartilage under the microscope one can see that there is present a second phase a precipitate which stains with silver nitrate. Presumably this is calcium phosphate and it precipitates in the form of very small very uniform balls. It is apparent that what has happened is that calcium has been taken up by ion exchange and calcium phosphate has been precipitated when this impregnated sample was submerged in concentrated phosphate solution. This precipitation has removed the calcium from the chondroitin sulfate and the cartilage is again able to bind additional calcium. This process can be repeated until a large amount of mineral accumulates whereupon the stoichiometric relations break down.

The Distribution Coefficient of an Ion Exchange Reaction

The distribution coefficient K_d of an ion exchange reaction involving a radioactive ion in which counts are proportional to mass may be defined as follows[105]

(1)

$$K_d = \frac{\text{counts in solid}}{\text{counts in solution}} \bullet \frac{\text{volume of solution}}{\text{mass of solid}}$$

It is thus possible to test a material for ion exchange properties by changing either the volume of solution or the mass of material used and determining the K_d In the following experiment a 0 01 M veronal buffer (pH 7 2) containing carrier free radioactive Ca^{45} and enough sodium chloride to make the ionic strength equal to 0 16 (approximately that of mammalian blood) was used Several 10 cc aliquots of this solution were equilibrated with amounts of sodium saturated cartilage of *Preparation A* varying from 30 to 1000 mg The solution was counted before and after the experiment the counts in the solid being determined by difference In all but the 30 and 1000 mg samples the ratio of counts in solid to counts in solution was within the range 0 6 to 1 7 A change in pH during equilibration was observed It was later shown to be toward a pH of about 5 4 regardless of whether the initial pH was above or below this value A similar phenomenon was observed by Freudenberg and Gyorgy [106] It is probably due to the buffering action of the protein in the cartilage The constancy of the K_d values obtained (Table III) lends support to the view that it is indeed an exchange reaction

The reaction involved in the exchange may be assumed to be

(2)

$$Ca^{++} + 2NaR \rightleftharpoons CaR_2 + 2Na^+$$

where *R* refers to the cartilage If radioactive carrier free Ca^{45} is used and it is assumed that the mole fraction is proportional to activity and that the amount of calcium cartilage complex present at equilibrium is much less than the amount of sodium cartilage complex present considerations of

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[105] Schubert J Russell E R and Myers L S Jr Dissociation Constants of Radium Organic Acid Complexes Measured by Ion Exchange *J Biol Chem* 185 387 398 (1950)

[106] Freudenberg E and Gyorgy P The Binding of Calcium by Animal Tissue *Biochem Z* 110 299-305 (1920)

TABLE III

Constancy of K_d With Varying Mass of Cartilage

Weight of Cartilage	K_d	Final pH
(mg)		
30	5.1×10^{-2}	7.15
100	5.9×10^{-2}	6.98
150	5.5×10^{-2}	6.65
200	5.6×10^{-2}	6.45
250	5.2×10^{-2}	6.18
300	5.6×10^{-2}	6.02
1000	4.7×10^{-2}	5.58

mass action allow the derivation of the equation [107]

(3)

$$K'_a = \frac{N_{CaR_2}}{C_{Ca^{++}}} \bullet \frac{\gamma^4_{NaCl} C^2_{Na^+}}{\gamma^2_{CaCl_2} N^2_{NaR}} ,$$

where N is the number of moles, γ is the activity coefficient, and C represents concentration in moles per liter.

Since

(4)

$$\frac{\text{Counts in solid}}{\text{Counts in solution}} = \frac{N_{CaR_2}}{N_{Ca^{++}}} = \frac{N_{CaR_2}}{C_{Ca^{++}} V} ,$$

where V is the volume of solution, *Equation 1* may be written

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[107] Boyd, G. E., Schubert, J., and Adamson, A. W. The Exchange Adsorption of Ions from Aqueous Solutions by Organic Zeolites. I. Ion-exchange Equilibria. *J. Am. Chem. Soc.* 69: 2818-2829 (1947).

(5)

$$K_d = \frac{N_{CaR_2}}{C_{Ca^{++}} M},$$

where M is the mass of solid, or

(6)

$$\frac{N_{CaR_2}}{C_{Ca^{++}}} = K_d \bullet M$$

If this is substituted in *Equation 3*, the following expression is found

(7)

$$K_a = K_d \bullet \frac{\gamma^4_{NaCl} C^2_{Na^+}}{\gamma^3_{CaCl_2}} \bullet \frac{M}{N_{NaR}}$$

Since the final factor is the molecular weight and is constant, it may be in corporated into the equilibrium constant

(8)

$$K_a = K_d \bullet \frac{\gamma^4_{NaCl} C^2_{Na^+}}{\gamma^3_{CaCl_2}}$$

The assumed exchange reactions may thus be tested by determining the constancy of K_a'' at various sodium concentrations

The above derivations involve the assumption that the activity coefficient of an ion in a solution of other ions is the same as though the total ionic strength were due to the ion in question This is usually assumed to be true and has been demonstrated experimentally in solutions containing relatively high concentrations of the ions involved Thus, the activity coefficient of the calcium chloride in *Equation 8* was calculated by using the concentration of sodium chloride There are, however, indications that this assump-

tion is not valid for an ion in very low concentration such as a carrier free radioactive tracer in which the bulk of the ionic strength is made up by other ions.[107] Feldman and Havill[108] have shown that the activity coefficient of beryllium ions is constant when the concentration is varied from 10 to 10^{-4} M and the ionic strength changes from 0.1 to 0.4. If the activity coefficient of the radioactive calcium in *Equation 8* is considered constant, the equation may be written[109]

(10)

$$K_a = K_d \bullet \frac{\gamma_{NaCl}\, C^{s}_{Na^+}}{\gamma^{s}_{KCl}}$$

Five samples of sodium saturated cartilage of *Preparation A* were equilibrated with solutions similar to those used previously except that the sodium concentration varied from 0.129 to 0.269 M. The ratio of counts in solid to counts in solution was kept within the range 0.9 to 1.4 by varying the mass of cartilage used. The K_d values obtained and calculated K_a'' and K_a values are given in Table IV.

The Dissociation Constant of the Chondroitin Calcium Complex

Apart from the actual values of K_a, it was of interest to test the behavior of the cartilage when placed in a solution of mixed ions approximating physiological conditions. Following immersion in a synthetic ultrafiltrate of plasma there was no phosphate uptake and the cartilage became only half saturated with calcium. I do not think that it is fortuitous that the cartilage is but half saturated with respect to calcium under physiologi

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[108] Feldman, I., and Havill, J.: Unpublished data.

[109] *Equation 8* may be written as follows:

(9)

$$K_a = K_d \bullet (\gamma^{s}_{NaCl}\, C^{s}_{Na^+}) / (\gamma_{Ca^{++}}\, \gamma^{s}_{Cl})$$

Since $\gamma^{s}_{KCl} = \gamma_{K^+} \bullet \gamma_{Cl}$ and assuming

$\gamma_{K^+} = \gamma_{Cl}$ then $\gamma^{s}_{Cl} = \gamma^{s}_{KCl}$

If this is substituted in *Equation 9* and the activity coefficient of calcium is incorporated in the equilibrium constant, *Equation 10* is obtained.

TABLE IV

Equilibrium Constants at Various Sodium Concentrations

Sodium Concentration	K_d	K_a	K_a
(M)			
0.129	8.5×10^{-2}	2.3×10^{-3}	8.3×10^{-4}
0.142	6.7×10^{-2}	2.2×10^{-3}	7.8×10^{-4}
0.161	5.6×10^{-2}	2.5×10^{-3}	8.4×10^{-4}
0.183	4.4×10^{-2}	2.5×10^{-3}	8.4×10^{-4}
0.269	2.7×10^{-2}	3.5×10^{-3}	10.5×10^{-4}

cal conditions. It is a strange coincidence that the dissociation constant should be of such a value to give an equal distribution of calcium between the solid and the solution phases. I am more inclined to think that chondroitin sulfate may act as a regulating compound; if the concentration of calcium is raised slightly, cartilage will take up the excess; if the concentration of calcium is lowered slightly, then calcium will dissociate from the resin. Thus chondroitin would act as a stabilizing influence with respect to the calcium ion concentration in its immediate surroundings.

It should be emphasized in this connection that our preparation represents a very special case. It is perfectly possible that cartilage taken from other parts of the skeleton or cartilage subjected to the different chemical manipulations may well have an effective dissociation constant quite different from that observed in our experiments. If this proves to be true all of my remarks concerning chondroitin as a regulating compound may be irrelevant.

The Effect of pH on the Dissociation Constant of the Chondroitin Calcium Complex

It was also of interest to determine whether or not the dissociation of the complex between cartilage and calcium was altered by variations in pH. At the time of these experiments I was entertaining the idea that chondroitin sulfate might be one of the vital factors concerned with calcification by providing a condition whereby a slight change in pH could release a great deal of calcium from the combined form. Such a scheme would provide a very favorable mechanism for the initiation of calcification. For this reason a group of samples was equilibrated with solutions essentially the same as before, except that they varied in pH. The values of K_d obtained are plotted against the equilibrium pH in Figure 26. Also in Figure 26

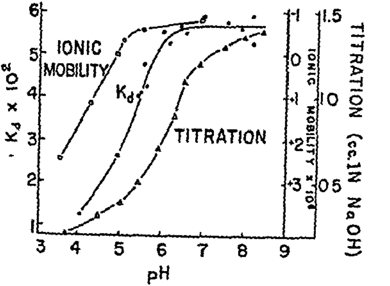


Fig 26 The Variation of K_d with pH

For purposes of comparison data on the ionic mobility (after Partridge[110]) and a titration curve of the protein component of cartilage are included

are the results obtained by the titration with sodium hydroxide of an extract of our cartilage preparation made by boiling one gram of the material with 100 cc of water for 30 minutes For further clarification of the results the ion mobilities of cartilage protein as determined electrophoretically by Partridge[110] are also presented in Figure 26

There are two points of interest in the graph obtained First you will note that, within the error of our reproducibility there is no variation in K_d throughout the physiological range in pH from 6 5 to over 8 Second the K_d rapidly falls as the pH is lowered below the isoelectric point of the protein compound Our interpretation of these results is that salt formation takes place between the protein moiety and chondroitin sulfate As the pH is lowered, the negative charge on the protein becomes less and less favoring competition with the calcium ion It would appear if I interpret Dr Meyer s results correctly, that the combination between chondroitin sulfate in this preparation is purely ionic in character or at least primarily ionic in character

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[110]Partridge S M The Chemistry of Connective Tissues I The State of Combination of Chondroitin Sulfate in Cartilage *Biochem J* 43 387 397 (1948)

In summary I would like to emphasize that three conclusions may be drawn from these studies 1) Of the two groups of investigators[103 104] that studied this system previously Freudenberg and Gyorgy[103] were the more correct Chondroitin sulfate in cartilage combines with cations—not with anions—by a process of ion exchange 2) The dissociation constant of the particular preparation was such that under physiological conditions the chondroitin was only half saturated with respect to calcium 3) The dissociation of the chondroitin calcium complex is unaltered by variations in pH throughout the physiological range of 6 5 to 8

Conference Discussion

Fremont Smith What was the concentration of calcium in your ultra filtrate?

Neuman About four or five milligrams per cent

Meyer In your original experiment when you treated your cartilage with Sequestrin did you get any protein coming out in the effluent solution?

Neuman Unfortunately we have no analyses on the original preparation

Meyer This seems to me an important question which comes out of the discussion here namely in what combination is the chondroitin sulfate present in the cartilage or cartilage product? I don t know data on sodium potassium calcium and so on in cartilage but I have the impression that there are not enough inorganic cations to give the necessary equivalents for both the sulfate and the carboxyl groups of chondroitin sulfate

Neuman That seems very reasonable

Gross Dr Neuman do you think perhaps you might be putting a little too much emphasis on this idea of physiological pH? After all what is the pH around the cell right in the vicinity of the cell surface? Do we know that it does not go down to 3 for example? Danielli and Davies[111] in a recent discussion on reaction at interfaces pointed out that the pH and the ionic strength at surfaces may be markedly different than in the bulk phase and that the pH can vary as much as several units at a surface as compared with the bulk I wonder if physiological pH necessarily covers all areas of the tissue and if perhaps in cartilage the pH at surfaces of fibrils and cells may vary considerably from that of the whole tissue

nate the problem But I would say this there are only two places in the body where I know the pH is below 6 and can be well below 6 One is in the lumen of the tubule of the kidney and the other is in the stomach I have been very interested in the study of carbonic anhydrase and many papers have shown this enzyme to be intimately associated with the secretion of hydrogen ions I don t know whether anyone has studied cartilage cells for carbonic anhydrase

Sobel Pierce[112] many years ago studied the pH of rachitic and normal epiphyseal cartilage He found it not very different from blood pH (7 37 to 7 40)

Neuman That is my impression, but you can always argue that if you cut it a little finer, get within the cell, the pH is different there

Shorr Chambers[113] has determined the pH within skeletal muscle cell for us and finds it to be about pH 6 8 I am referring to the intracellular pH—that is, the cytoplasmic pH

Meyer We found the ratio of total nitrogen to hexosamine the same in cartilage as in artificial salts of gelatin with chondroitin sulfate This finding was one of the reasons for our suggestion that chondroitin sulfate was present in native cartilage in salt linkage with protein However this presumably would require in cartilage a protein of an isoelectric point of above 8, or whatever your highest figure is

Neuman No, I don t think so I think that you could have a protein with an over all negative charge and still it would be able to bind negative groups with its basic ionized groups I don t believe you are at a loss for an explanation

Meyer You would expect a competition between all ions present In the artificial mucoids we get the theoretical and constant amount of chondroitin sulfate bond if we lower the pH below the isoelectric point of the protein You have to ionize the free NH_2 groups of the protein in order to get the expected two equivalents of acid bond one from the sulfate the other from the COOH group

Hastings May I ask a question and make an observation? The question is have you ever set up tendon or tendon fractions in a column like this—an ion exchange column? Do you know anything about the calcium binding?

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[112] Pierce J A The Reaction of the Epiphyseal Cartilage in Normal and Rachitic Rats *J Biol Chem* 124 114 124 (1938)

[113] Chambers, R Personal communication

Neuman No

Armstrong The point I am interested in is whether or not the bone cartilage is unique in this regard

Hastings Yes The reason I asked is that I looked up a series of unpublished data obtained by Dr C B Mueller some years ago on calcium analyses of tendon He did quite a large series Roughly, it works out to be only 1/100th of a millimole of calcium per gram of dry tendon solids, so that that is 1/1000th of your figure for the maximum binding of cartilage I have no idea what the maximum binding of tendon would be

Neuman The point is this the maximum binding may not correlate very well in tendon with its cation content It would depend on the dissociation constant You will find that K_d can be altered both by the configuration of the sulfate and by the nature of the protein component

Hastings The other point I want to remind you of is that noncalcified cartilage has a very high sodium content It is quite beyond the amount of sodium that you would expect to be there in solution

STUDIES ON THE 'LOCAL FACTOR' OF CALCIFICATION [111]

ALBERT E SOBEL

om the Department of Biochemistry the Jewish Hospital of Brookl n Brooklyn N Y

Armstrong I think Dr Sobel has some work that fits in well with th vious discussion

Sobel I think it will fit in with what has been said I m ght add that r new work on the nature of the 'local factor has been largely stimulated the discussions at the previous meetings of this conference which I have d the privilege of attending When I saw Dr Newman s paper I ought we were hand in glove and beginning to get together but I am n t sure about it after hearing today s discussion

The Differences and Similarities Between Strontium Rickets and Calcium Rickets

First I would like to summarize the differences and similarities between ontium rickets and calcium rickets When strontium carbonate replaces cium carbonate in a rickets producing diet one develops a rickets which histologically similar to calcium rickets but which does n t respond to amin D, furthermore such rachitic cartilage does not calcify under con ions suitable for calcification *in vitro* of the control cartilage When ontium carbonate is replaced by calcium carbonate in the diet that is en the animals are first given strontium carbonate the bones heal *in vi o* h vitamin D and *in vitro* in calcifying solution, in a manner similar to t of the control cartilage Moreover, strontium rach tic bones after aking with calcium chloride will calcify *in vitro* in a manner similar to ntrol rachitic bones (referred to as calcium rachitic bones) [116 1 0]

This work was supported by the National Institute of Dental Re earch of the tional Institutes of Health U S Public Health Service Earlier phases of this dy were supported by the Williams Waterman Foundation

Boyd E S and Neuman W F The Surface Chemi try of Bone V The I n nd ng Properties of Cartilage *J Biol Chem* 193 243 2ɔ1 (1951)

Sobel A E ... of Incurable R ckets I ... aling *Proc Soc Exp r* ...

... the Injury to the Calc ... 2640-2645 (1935)

Sobel A E, Cohen J and Kramer B Phosphatase Activity and Calcification ...

... cy *Conference on Meta* ...

... ati n IV Infl ence of ... *oc Soc Exper Biol &*

Bartter Is the 'control cartilage' produced with a low phosphate, high calcium diet?

Sobel Yes a low phosphate, high calcium diet

The Survival and the Protection of the Calcifying Mechanism

We made some observations with respect to the *survival of the calcifying* mechanism which are summarized as follows

olution with
M per liter
n is similar
n the same
solution

Control sections do not calcify when kept for 18 hours in a solution with a low Ca × P *product of 20 (Ca = 10 mg % P = 2 mg %)* Such sections will not calcify *in vitro* in the next 48 hours when transferred to the solution with the higher product of Ca × P = 60 and Mg = 0 75 mM per liter

From these findings we drew the conclusion that strontium in the presence of a solution containing the magnesium ion combines with a factor in the matrix essential for calcification and this combination protects the calcifying mechanism These observations led to the concept that one of *the integral elements of calcification is the combination of calcium with* something in the matrix with which strontium can compete, in fact we know now that strontium itself can be a mineralizing ion [121 122] and Marks and Shorr[123] have presented a very nice paper along these lines here two years ago

The Destruction of the Calcifying Mechanism and Its Prevention

Table V shows some other experiments that derived from these thoughts concerning the disappearance of the calcifying solution It gave us a standard healing of 2 (++++) I might add that 2 (++++), at which we always worked is about one half the maximum healing so that we could detect either more healing or less healing When one gets maximum healing in the controls as was the case in the *in vitro* calcification studies reported by *Marks and Shorr*[123] *it is very difficult to detect the effect of* interfering ions as the solution may have a higher Ca × P product than

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[121] Robison R and Rosenheim A H Calcification of Hypertrophic Cartilage *In Vitro Biochem J* 28 684 698 (1934)

[122] Robison R Law K A O and Rosenheim A H Deposition of Strontium Salts in Hypertrophic Cartilage *In Vitro Biochem J* 30 66 68 (1936)

[123] Marks P A and Shorr E Factors Which Regulate the Deposition of Calcium and Strontium in Rachitic Cartilage *In Vitro Trans Macy Conference on Metabolic Interrelations* 2 191 202 (1950)

TABLE V

The Prevention of Disappearance of the Calcifying Mechanism

Experiment	Treatment	Degree of Calcification
Control	Rachitic bone section placed in Calcifying Solution *	2 (++++)
Disappearance	Rachitic bone section placed in Basal *Salt Solution*** for 7 hours at 37° C and then placed in Calcifying Solution	0 (0) to trace
Prevention	Rachitic bone section placed in Basal Salt Solution containing in addition 10 mg % Ca^{++} or 10 mg % Sr^{++} for 7 hours at 37° C and then placed in Calcifying Solution	2 (++++)

*Calcifying Solution per liter 97 mM Na^+ 5 mM K^+ 75 mM Cl^- 22 mM HCO_3^- 2.5 mM Ca^{++} (10 mg %) and 1.6 mM PO_4 (5 mg % P) pH 7.3 temperature 37° C.

**Basal Salt Solution per liter 97 mM Na^+ 5 mM K^+ [illegible] mM Cl^- [illegible] 22 mM HCO_3^-

the least amount required for maximum calcification so that interference cannot be detected

When one places a rachitic bone section in the so called Basal Salt Solution the composition of which is indicated in Table V for about 7 or 12 hours and then tries to calcify *in vitro* subsequently no calcification will take place under the conditions which will produce 2 (++++) healing in the control section But if one puts such a rachitic bone section in the Basal Salt Solution containing in addition either calcium or strontium ions at a concentration of 10 mg % for 7 hours and then places it in the calcifying solution one obtains as good calcification as in the original control Thus the presence of a small amount of calcium or strontium ion prevented the disappearance of the calcifying mechanism In contrast when we added beryllium there was simple inactivation in two hours However in the case of magnesium and barium there was no prevention of the disappearance of the calcifying mechanism It is of interest that when we added to the Basal Salt Solution not only beryllium but phosphate at the same time in amounts that did not show a visible precipitate there was no inactivation of subsequent calcification by beryllium in two hours These findings may

help in interpreting the data of Gutman and Yu[124] on beryllium inhibition in the presence of phosphate From these studies we drew the tentative conclusion that calcium and strontium must combine with some factor in the cell and that the combination prevents the destruction of the calcifying mechanism

The Reversible Inactivation of the Calcifying Mechanism

As far as the reversible inactivation of the calcifying mechanism is concerned I would like to give you briefly a summary of what I presented in a more extended form at this conference two years ago [119] The essential data are shown in Table VI Here again we have adjusted the control

TABLE VI

The Reversible Inactivation of Calcification In Vitro

Experiment	Treatment	Degree of Calcification
		(mean)
Control	Rachitic bone section placed in Calcifying Solution *	2 (++++)
Inactivation	Rachitic bone section shaken for two hours with the chloride salts of the cation mixtures shown below then *washed with distilled water and then* placed in Calcifying Solution 1) 150 mE/L Ca++ and 0 1 mE/L Be++	0 (0) or 1 (+)
Reac	cation mixtures shown above then washed with distilled water then shaken for one hour with 150 mE/L *Ca++ as the chloride salt* and finally placed in Calcifying Solution	3 (++++) to 4 (++++)

*Calcifying Solution per liter 97 mM Na+ 5 mM K+ 75 mM Cl− 22 mM HCO− 2 5 mM Ca++ (10 mg %) and 1 6 mM PO (5 mg % as P) pH 7 3 temperature 37 C

[124] Gutman A B and Yu T F A F... ...erat on ... s of Beryllium Salts on *In Vitro* Calcificat on of ... M... *on Metabolic Interrelations* 3 90 104 (1951)

conditions so as to give intermediary healing which we call 2 (++++) Inactivation of the calcifying mechanism takes place when one shakes the rachitic bone section for two hours in the presence of a mixture of 150 milliequivalents per liter (an arbitrarily chosen amount) of calcium ion and a second ion which we regard as a competitor Subsequent to shaking the sections with any of these mixtures one obtains in them either a trace or no calcification after exposure to the calcifying solution The interesting fact was that strontium appeared to be a relatively poor inhibitor As you will see later, this was due to the fact that at the time we ran these experiments we had removed magnesium from the solution that was used for *in vitro* calcification

Following inactivation if one shakes the bone section with calcium chloride washes it with distilled water and then places it in the solution employed for calcification *in vitro* one obtains a calcification which is actually more intense than the initial calcification obtained with the control bone section This again we interpreted to mean that the combination of calcium with something in the matrix is a necessary preliminary step to ossification and of course the higher the degree of saturation with calcium the more intense the calcification By shaking the bone sections with calcium chloride solutions of greater than physiological strength we made it possible for the factor in the matrix to become combined with calcium to a much greater extent than when exposed to the five milliequivalents of calcium or less present in the usual *in vitro* solutions or *in vivo* fluids

The Synergism Between Magnesium and Strontium in Inhibiting *In Vitro* Calcification of Calcium Rachitic Bone

I have two divergent studies arising from this experiment I would like first to refer to the interesting investigation of Dr Shorr and his colleague[13] in which they demonstrated that when one puts strontium ions in a solution in which calcification would normally take place the strontium ions in no way interfere with *in vitro* calcification The difference between their procedure and ours in the past was that they omitted the magnesium just as we have done more recently Therefore we proceeded to examine whether or not the magnesium might be a critical factor in explaining the difference

A typical experiment is shown in Table VII[1,9] In this we have the concentration of strontium and of magnesium expressed thus ... in milli moles per liter and the degree of calcification with different ... ium rachitic bone sections each set representing a series of ... r tibiae were cut into four slices and several ... were divided among these experiments The ... follows 1) with strontium and magnesium

TABLE VII

The Influence of Strontium and Magnesium Ions on Calcification *in Vitro*

Sr	Mg	Degree of Calcification (Mean)*	
(mM/L)	*(mM/L)*	*(Ca × P = 40**)*	*(Ca × P = 70**)*
0.58	0.75	none	none
0.58	0	1.1 (++++)	—
0	0.75	1.1 (++++)	—
1.33	0	—	2.3 (++++)
0	1.33	—	1.5 (++++)
0	0	2 (++++)	2.7 (++++)

*As evaluated under the low power microscope
**In calculating the Ca × P product the Ca in mg % and the inorganic PO as P in mg % are used The Ca was 10 mg % in these experiments

of calcification in the system 2) with strontium in amounts equal to the sum of the magnesium and the strontium together there was a diminution of calcification compared with the calcification of the control but this diminution was rather small in line with Dr Shorr's experiments,[1 ?] and 3) with magnesium in amounts equal to the sum of both substances together there was again a diminution of calcification compared with the calcification of the control but compared with the complete inhibition of strontium and magnesium together the diminution was very small These results imply that magnesium is essential for the inhibition by strontium, in fact that magnesium will act as a synergist to the inhibition by strontium We may assume that the *local factor* has two types of combining groups and if one group is bound let us say by magnesium and this binding *does not interfere too much with calcification* the second group is the only one left open for combination with strontium

The Interaction of Magnesium and Strontium in Inhibiting *In Vitro* Calcification of Strontium Rachitic Bone

These results prompted us to reinvestigate the mineralization *in vitro* of bones from animals suffering from rickets due to strontium As can be *seen in Table VIII strontium rachitic sections* placed *in* the calcifying solution without magnesium calcify to the same extent as do the control calcium rachitic sections However when *magnesium is placed in the solution no calcification in vitro* takes place in the strontium rachitic sections whereas in the same solution one observes excellent *calcification in vitro*

TABLE VIII

Influence of Magnesium Ions on the Calcification *In Vitro* of Strontium Rachitic Bones

Experiment	Mg	Degree of Calcification (Mean)*	
		Sr Rickets *(Experimental)*	Ca Rickets *(Control)*
	(mM/l)		
Control	0	2 (++++)	2 (++++)
Inactivation	1	0 (0) to trace	2 (++++)
Reactivation**	0	3 5 (++++)	3 5 (++++)
Reactivation**	1	3 5 (++++)	3 5 (++++)

*As evaluated under the low power microscope

**Rachitic bone sections shaken 2 hours with a solution containing 75 mM l   1 $CaCl_2$ prior to incubation with the Calcifying Solution (see Table V) to which the magnesium was not or was added as indicated

in the control calcium rachitic sections Thus again it appears that the injury to the calcifying mechanism in rickets due to strontium is associated with the presence of magnesium ions in the calcifying solution

In other experiments strontium rachitic bone sections were shaken in the manner previously described[119, 125] for the reactivation of the calcifying mechanism and then exposed to calcification *in vitro* both in the presence and in the absence of magnesium As can be seen in Table VIII the magnesium no longer had an injurious effect on the calcifying mechanism From this it may be deduced that the injury to the calcifying mechanism in strontium rickets is due to the interaction of the strontium and the magnesium ions In the naturally occurring body fluids one usually finds magnesium so the chances are that the reason that there is no response in strontium rickets to vitamin D *in vivo* is that *in vivo* the magnesium ions have interacted with the strontium ions and injured the calcifying mechanism

The Synergism Between Magnesium and Fluoride and Between Magnesium and Cyanide in Inhibiting *In Vitro* Calcification of Calcium Rachitic Bone

Before we speculated further, we were reminded of the controversy discussed at these conferences on the influence of fluoride and cyanide ions

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[125] Kel A E Sobel S and Hanok A The Reversible Inactivation of Calcification *In Vitro Proc Soc Exper Biol & Med* 72 68 72 (1949)

on calcification *in vitro* of calcium rachitic bone sections Robison and Rosenheim,[121] and Gutman and his colleagues[126] reported that these ions inhibit calcification *in vitro* Other groups, including McLean, *et al*,[127] and our group[128] were unable to confirm this Dr Shorr and his group[123] have not been able to repeat the cyanide inhibition but I am not certain whether or not they were able to confirm the fluoride inhibition

Shorr Yes, we found inhibition of calcification *in vitro* with fluoride

Sobel We found that we could not obtain inhibition either by fluoride or cyanide in the concentration presented in Dr Gutman's paper, in the absence of magnesium However, we could obtain fluoride inhibition if we allowed the calcification to proceed for only five hours In other words, in five hours there was a manifest difference between the fluoride treated sections and the controls, but in 18 hours, our usual period, there was no difference, in fact, if the fluoride were raised sufficiently, there appeared to be an actual enhancement of calcification That this was not a simple artefact was proved by inactivating in two different ways, namely, by acid treatment and by beryllium ion treatment Subsequent to that, fluoride did not enhance calcification,[128] in fact, no calcification took place at all

TABLE IX

The Influence of Fluoride, Cyanide and Magnesium Ions on Calcification *In Vitro*

F	CN	Mg	Degree of Calcification*
	(millimoles / L)		(mean values)
0	0	0	2 (++++)
0	0	1 0	1 5 (++++)
1 0	0	0	3 (++++)
1 0	0	1 0	none
0	1 0	0	2 (++++)
0	1 0	1 0	trace

*As evaluated under the low power microscope

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[126]Gutman, A B, Warrick F B, and Gutman E B Phosphorylative Glycogenolysis and Calcification in Cartilage, *Science* 95 461-462 (1942)

[127]McLean, F C, Lipton, M A Bloom, W, and Barron E S G Biological Factors in Calcification in Bone, *Trans Conference on Metabolic Aspects of Convalescence* 14 9 19 (Nov 1946)

[128]Goldenberg H, and Sobel, A E Calcification. V Influence of Fluoride and Cyanide Ions in the Presence and Absence of Magnesium *Proc Soc Exper Biol & Med* 78 719 723 (1951)

Table IX shows not all of the experiments but only typical ones indicating the fluoride cyanide and magnesium inhibition We tried these series at several different concentrations If you have any questions I can give additional data later In the control sections which received no fluoride cyanide or magnesium there was a 2 (++++) healing which I call to your attention again is half the maximum healing in our scale Magnesium alone produced some inhibition but not what you would call a marked inhibition Fluoride alone produced enhancement which was repeatedly observed with a concentration of one millimole per liter but not with a concentration of a tenth of a millimole per liter When fluoride and magnesium were used together there was complete inhibition (no healing) This was true even when we used one millimole of fluoride per liter This provides an interesting contrast since the same concentration of fluoride when used alone enhances the calcification and when used with magnesium inhibits it

With cyanide the story was essentially the same Cyanide itself in a concentration of one millimole per liter and in magnesium free solution permitted healing that was every bit as good as the control On the other hand in the presence of the magnesium ion cyanide was a very good inhibitor In the cyanide magnesium sections we often found traces of calcification which were absent in the fluoride magnesium sections so the fluoride and magnesium combination under these conditions produces a somewhat stronger inhibition

The Effect of 'Mild and of Drastic Treatment on the Reversible Inactivation of the Calcifying Mechanism

We are left with a puzzle The tempting conclusions are obvious but perhaps Table X will eliminate some of them Here we followed some of the experiments of Dr Waldman[129] from Dr McLean s laboratory with modifications We employed the reversible inactivation scheme with calcium chloride (as in Table VI) and treated bone sections by what we called drastic and mild means For example if one shakes for two hours with methyl alcohol and subsequently shakes for one hour with calcium chloride there is no calcification If one shakes for two hours with xylene there is no calcification but if this treatment is followed by shaking for one hour with calcium chloride there is good mineralization The next experiment heating bone sections in distilled water at 97° C is similar to the studies that Dr Waldman presented here two years ago (See Waldman[129] Table XXIII) When we attempted to calcify *in vitro* rachitic

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[129]Waldman, J Effect of Inactivation of Enzymes on Calcification of Cartilage *In Vitro Trans Macy Conference on Metabolic Interrelations* 2 203 220 (1950)

TABLE X

Inactivation of the Calcifying Mechanism by Mild and Drastic Treatments

Inactivation		Reactivation	
Treatment	Degree of Calcification	One Hour Shaking With CaCl	Degree of Calcification
	(mean)	*(mE/L)*	*(mean)*
Shaken for two hours in methyl alcohol	0 (0)	150	0 (0)
Shaken for two hours in xylene	0 (0)	150	2 5 (++++)
Heated for one hour in distilled water at 97° C	0 (0)	150	2 (++++)
Heated for one hour in 150 mE/L of CaCl at 97° C	0 (0)	150	0 (0)

sections that had been heated for one hour at 97° C in 20 cc of distilled water, we obtained no calcification When we shook such heated sections with calcium chloride however we obtained *in vitro* calcification Now here is the interesting point when we heated the rachitic sections in a solution with 150 milliequivalents of calcium chloride instead of in distilled water and then tried to calcify them there was no calcification We even shook them with calcium chloride but there was no calcification In other words, heating with the calcium chloride destroyed the calcifying mechanism

Now with these two experiments we have a scheme for exploring the mechanism of calcification Whatever does not survive heating in distilled water such as many of the enzymes may be excluded as part of the minimal system required for calcification and thus we may systematically examine the various postulated enzymes and establish later whether they survive heating at *97° C On the other hand the factors that are present* in the sections heated in distilled water at 97° C and are not present (destroyed or extracted) when the bone is heated in calcium chloride may well give us clues to the minimal mechanism required for calcification

The Competition Between *Chondroitin Sulfate Inhibitors and* Calcium for the Calcifying Mechanism

An important question has been raised particularly by the presentations today by Dr Follis, Dr Dziewiatkowski and Dr Neuman and also by

the paper of Rubin and Howard[150] which was presented here two years ago. Is chondroitin sulfate or some related compound the key to the understanding of the local mechanism of calcification? Rubin already has suggested that our experiments on the reversible inactivation of calcification *in vitro* by means of cations may be explained by postulating an acid mucopolysaccharide as the target in the calcifying matrix. One is tempted to think of the possibility of a substance possessing an ion-exchange mechanism and, in view of all the other information, one is again tempted to think of chondroitin sulfate.

I shall close my presentation with the results of a preliminary attempt at an experiment to show whether or not chondroitin sulfate or a related compound is the calcifying factor. The ultimate solution will be, of course, to put something into a test tube and produce or show that this something or combination of factors will produce calcification. In the meantime, we thought that if we worked with inhibitors that are supposed to have as a specific target a mucopolysaccharide sulfate like chondroitin sulfate, we might get somewhere. Protamine is one of the compounds that combines with chondroitin sulfate rather avidly. Similarly, toluidine blue combines with the mucopolysaccharide, heparin, and we know that calcium and toluidine blue compete to combine with heparin. Possibly acriflavin behaves in the same way. Thus far, we have done experiments only with protamine-calcium mixtures. We put the rachitic bone sections in a basal solution containing a certain amount of protamine and found that subsequently calcification is inhibited. We cannot even restore the calcifiability with calcium chloride. Then we used calcium protamine mixtures, and we found that after treating the sections with such mixtures we were able to obtain calcification, depending on the calcium to protamine ratio in the basal salt solution. In other words, it appears that calcium and protamine are competitors for the calcifying mechanism; that is, calcium is the activator, protamine is an inhibitor, and in this case a compound like chondroitin sulfate may be the specific target.[151]

I must make one point about these studies, that is, that the subsequent calcification was always subsurface in character. On the surface itself one could not see anything. By clearing the specimen, one could see oriented calcification beneath the surface, and when other combinations of calcium

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[150] Rubin, P. S., and Howard, J. E.: Histochemical Studies on the Role of Acid Muco-Polysaccharides in Calcifiability and Calcification. *Trans. 2nd Conference on Metabolic Interrelations* 2: 155-166 (1950).

[151] Harris, A. L.: The Biochemistry of Calcification. Thesis submitted in partial fulfillment of the requirement for the degree Master of Science in Chemistry, Polytechnic Institute of Brooklyn, Feb. 1952.

and protamine were employed (*with lower ratios of calcium to protamine*) no such subsurface calcification could be obtained

Therefore the reason that I think this ties in with Dr Neuman s experiments is that he found that chondroitin sulfate takes up quantitatively barium sodium and calcium and sodium is one of our inactivating competitors I wish he had studied beryllium just to complete the picture If we are both working on the same substrate perhaps we may propose a compound having properties similar to chondroitin sulfate as a factor in the local mechanism of ossification

Conference Discussion

Howard Dr Sobel was the heat at 97° C in the bottom of the column the same as in the top? You say it was still 97° C? What was the temperature of the calcium chloride?

Sobel We put the thermometer inside the column

Howard It was 97° at both?

Sobel The heat was about the same throughout

Armstrong It would be nice to consider that we know what the local factor is Dr McLean do you want to comment on this point at this time?

McLean Well I would like to comment on the fact that there is an important difference between Dr Sobel s material and Dr Neuman s material Dr Sobel is working with cartilage matrix that is destined to be calcified or easily calcifiable matrix Dr Neuman is working with hyaline cartilage which is not normally easily calcifiable The same point applies to the Freudenberg and Gyorgy work[13] They had the idea that there was something that reaches out into the blood and takes out calcium and concentrates it and therefore makes it easily available for precipitation with the phosphate which comes along later That theory has always suffered from the fact that they were working with a cartilage which is not calcifiable In the calcification process if the local factor is *chondroitin sulfuric acid* then there must be some *difference in* the chondroitin sulfuric acid that Dr Sobel is looking at and that *with which Dr Neuman is concerned* because there is a tremendous difference in the two substrates

Neuman As a matter of fact I believe I stated that I did not think chondroitin sulfate was a vital factor I considered it more of a regulating

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[13] *Freudenberg E* and *Gyorgy P* The Calcification Process in the Development of Bone *Ergeb inn Med u Kinderheilk* 24 17 28 (1923)

factor Initially, I held the view that chondroitin sulfate may be a template that it binds the cations in a spatial relationship which corresponds to the spatial arrangement in the apatite lattice Thus the calcium salt of chondroitin represented a calcium surface with which phosphate ions could collide to form the apatite lattice This is still a very attractive idea and it may well be that what is important is not just the chondroitin but the chondroitin protein combination In that case we could have differences from tissue to tissue I don t know enough about the protein present Also, this may explain perhaps the effect of heat If you form a salt with the chondroitin, that will affect its combination with the protein The protein may then denature and subsequently you cannot recalcify It seems to me that we are so far beyond the limits of our knowledge that conjecture at this point is only fun

Hendricks May I throw in a monkey wrench The monkey wrench I want to throw in is that I will attempt to interpret these phenomena tomorrow as not involving chondroitin sulfate at all but simply as involving the mechanism of growth of apatite as a crystal

Sobel In these series there is no apatite preformed

Hendricks But still it is a substance that does form isn t it?

Sobel Of course, I assumed that most of this group knows what is meant by *in vitro* calcification I didn t want to bore them with a description of it

Hendricks Maybe I don t know

Sobel At the Second Conference of this group I presented an illustration which shows what *in vitro* calcification is (see Sobel[119] *Figure 23*) In rachitic bone metaphysis there is proliferated cartilage and bare osteoid In this region there is no apatite that we can discern All new calcification takes place in this area (see Sobel[119] *Figures 24 to 26*) so here we are involved with the problem of how does the first crystal form rather than with the problem of what happens after it has formed

Hendricks The problems are not necessarily different

McLean Within a few micra of each other there are found tissues in which apatite will be laid down and tissues in which it will not

Shorr Could this difference be dependent upon the proximity to hypertrophic cartilage cells rather than to variations in the chondroitin sulfuric acid content?

McLean I don t know Of course there is another way of looking at this problem and that is to invert it and to ask why all of the tissues in

the body do not calcify One might assume that there is an inhibitor of calcification in most of the tissues, and that bone is one tissue without the inhibitor I think Dr Sobel has made a very important contribution by exploring the reversibility of inhibition of calcification This is sure to lead to a better understanding of some of these processes because when a reversible phenomenon is at hand, the opportunities of studying it are enormously improved

Neuman You have done a lot toward producing a better state of amity between investigators, anyway, by making *everybody* right (Laughter)

Copp I have a rather naive question If chondroitin sulfuric acid is postulated as the important 'local factor,' then why is the concentration of it in the osteoid matrix so small?

Meyer I wonder whether anyone knows which type of chondroitin sulfate is present in the area of calcification As far as I know, the only chondroitin sulfates characterized were those of hyaline cartilage and those of some connective tissue

Sobel *I must make it clear that I said there is "a compound like chon* droitin sulfate" somewhere in the calcifying preosseous matrix

Armstrong Dr McLean has a question concerning the physical state of the proteins of cartilage and bone matrix

McLean This is entirely in the nature of a comment I have regretted not hearing today anything about the physical state of the bone matrix or ground substance or whatever we have decided to call it, and particularly with reference to polymerization and depolymerization, and so on These are subjects that I have understood are coming into the picture in relation to calcification and I had hoped I would be enlightened about them today, *but I haven t heard anything*

Armstrong Does anyone wish to shed any light on this point?

Follis Would you elaborate what you mean by *'physical state'*?

McLean Well let s confine it for the moment to polymerization and *depolymerization*

Dallemagne After Dr Sobel s very interesting communication, I must tell you about some results obtained recently by Cartier in Paris These observations may be of some interest to Professor Gutman, too *Instead* of using rachitic rat epiphyses as has been done before, Cartier has studied *in vitro* the calcification of normal epiphyses of sheep embryos He observed[133] that calcification of this tissue is very limited in the presence

[133]Polonovski, M, and Cartier P On the First Biochemical Stage of Ossification *Compt rend Acad sc Paris* 232 119 121 (1951)

cartilage 35 milligrams per cent of ATP which compares with the amount of that compound in the normal kidney liver and brain This is a rather high concentration of organic phosphate In studying the prevention of the disappearance of the calcifying mechanism we found that the rate of disappearance of the ATP was slower when calcium or strontium ions were present (Table V) paralleling the prevention of the disappearance of the calcifying mechanism In fact the circumstantial evidence looked fairly good for ATP being one of the critical factors But when we heated bone sections in distilled water at 97° C and found apparent survival of the calcifying mechanism (Table IX) we also found practically no ATP Therefore while we were well aware of Dr Cartier s work[133 134] and were quite enthusiastic about it I did not refer to it I mention it now because it may be that these heating experiments are not significant and that ATP is still an important factor in the calcifying mechanism In other words if we ignore the fact that by heating a bone section in distilled water at 97° and following this with treatment with calcium chloride we can still obtain calcification *in vitro* the agreement between Cartier and the experiments just described might add circumstantial evidence for ATP as a critical factor in the system required for ossification At the moment I don t know how to interpret these data

Copp I would like to put forward a suggestion that the ATP may play a role in converting the preosseous matrix to calcifiable matrix but that the actual process of calcification may be independent of ATP

Dallemagne The discovery of the presence of ATP in bone by Cartier is very recent I have received no other observations from Cartier on that topic

Copp Professor Dallemagne if the uncalcified epiphysis from the embryo sheep is placed in a suitable medium does it require the presence of ATP or will it calcify without it?

Dallemagne Yes it requires the presence of ATP to calcify

Copp Then this might lend support to the idea that the ATP converts the cartilage to a form which can be calcified?

Dallemagne Yes but the ATP is active without the presence of other substances

Shorr This would mean that the ATP is not limited to the cell but is diffusely present throughout the preosseous tissue This would represent a very unusual biological situation

Dallemagne I have here the paper sent m[illegible] Cartier In the photographs of the epiphyses of sheep embryo [illegible] by [illegible] *od of*

von Kossa, only the slices which have been in contact with ATP give a a positive response. After contact with phosphoglyceric acid, glucose phosphate, or fructose phosphate, they do not contain more mineral elements than do the control slices in contact with PO_4 ions only.

Copp: Does the phosphate which is precipitated in the bone salt below the epiphysis come from the ATP or from inorganic phosphate?

Dallemagne: It comes from the ATP.

Copp: It might be that the ATP is providing a source of energy for conversion of the preosseous cartilage to a calcifiable form and that it also incidentally supplies phosphate.

Dallemagne: Yes, it supplies phosphate for the calcification.

Copp: The other sources of phosphate would not supply the energy to cause the change to a calcifiable form.

Shorr: Does anything happen in these particular preparations in the presence of inorganic phosphate? Calcification does take place with inorganic phosphate as well as with ATP, does it not?

Dallemagne: Yes, but not to the same extent, under the same conditions.

Sobel: Actually, the amount of new mineralization in Cartier's studies[133, 134] was about ten times as great with ATP providing the phosphate as when equivalent amounts of inorganic phosphate or organic phosphate were employed. In studying the *in vitro* calcification of embryonic sheep bones, Cartier measured the amount of newly formed mineral by direct chemical analysis. With ATP, new calcification seemed to be increased much beyond that found with other sources of phosphate.

THE RELATIONSHIP OF PARATHYROID EXTRACT TO THE PHOSPHATASES IN KIDNEY AND IN BONE

CHARLES D KOCHAKIAN

From the Section on Biochemistry, Oklahoma Medical Research Institute and Hospital, Oklahoma City, Oklahoma

Armstrong I understand that Dr Kochakian has some work to describe which has to do with bone Dr Kochakian, will you tell us about it?

Kochakian Part of the data I am going to present was given several years ago at this same conference [139, 140] I am going to talk about the relationship of parathyroid extract to the phosphatases in kidney and in bone These studies were stimulated as a result of discussions in this conference by Dr Albright The data I am to present have now been published in final form [141]

Chronic Experiments with High Doses of Parathyroid Extract

These experiments were carried out in female white rats (ranging in body weight from 84 to 121 gm) of the Wistar strain from our colony fed Purina dog chow *ad libitum* The regimen for the administration of the parathyroid extract[142] was designed to duplicate that of the experiments by Ingalls *et al* [143] The extract was injected subcutaneously into half of the rats at approximately 12 hour intervals and at 2 cc (200 units) per injection for two days The animals were then fasted for 24 hours, and autopsied at 60 or 66 hours after the first injection The results are presented in Figure 27 The high dose of parathyroid extract caused a marked loss in body weight, enlargement of the adrenals and also of the kidneys with intense calcification The rats showed prostration with suggestions of

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[139] Kochakian, C D The Effect of Parathyroid Hormone and Parathyroidectomy in Female Rats, *Trans Conference on Metabolic Aspects of Convalescence* 7 153 160 (1944)

[140] Kochakian, C D The Effect of Parathyroid Hormone on the Phosphatase Content of the Rat Kidney, *Trans Conference on Metabolic Aspects of Convalescence* 8 50 63 (1944)

[141] Kochakian C D, and Terepka A R Effect of Parathyroid Extract on Tissue Enzymes and Serum and Urinary Phosphorus *Am J Physiol* 165 142 148 (1951)

[142] The parathyroid extract was generously provided by Eli Lilly and Company

[143] Ingalls, T H, Donaldson, G A, and Albright F The Locus of Action of the Parathyroid Hormone Experimental Studies with Parathyroid Extract on Normal and Nephrectomized Rats *J Clin Invest* 22 603 608 (1943)

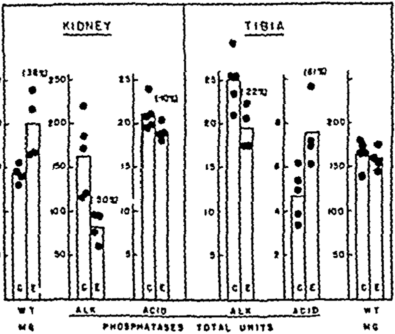


Fig. 27. The Effect of Parathyroid Extract on the Phosphatases of the Kidneys and of the Tibia of Female Rats.

Female rats weighing 84 to 121 grams were injected subcutaneously with 200 units of parathyroid extract (Lilly) twice daily for two days and were autopsied [illegible] hours after the first injection. The control animals gained an average of [illegible] grams and the experimental animals lost an average of 15 grams of body weight. The hormone caused calcification of the kidneys and an enlargement of the adrenal glands which averaged 81 per cent.

[Reproduced by permission from Kochakian, C. D. and Terepka, A. R.: Effect of Parathyroid Extract on Tissue Enzymes and Serum and Urinary Phosphorus. Am. J. Physiol., 165: [illegible] (April 1951).]

[illegible] and one rat died 18 hours after the fourth injection. Thus a very severe hyperparathyroidism was produced in these animals. Identical results have been reported by Moore.[141]

The alkaline phosphatase of the tibia was only slightly decreased, but the acid phosphatase was significantly increased. I would like to raise the question of whether or not this change in acid phosphatase is directly concerned with the dissolution of bone. I hope Dr. [illegible] will discuss this

[141] Moore, R. D. [illegible] Bone Marrow [illegible] Tissue Hyperemia [illegible] 36: [illegible] (1943).

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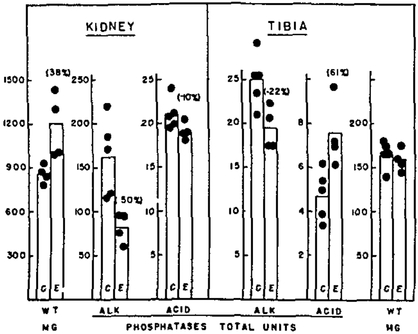


Fig 27 The Effect of Parathyroid Extract on the Phosphates of the Kidney and of the Tibia of Female Rats

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[144] Moore R D Calcification of Bone Marrow in Toxic Hyperparathyroidism *Am Arch Path* 36 51 57 (1943)

point It is now known that hyperparathyroidism will cause an increase in the acid as well as the alkaline phosphatases of the serum [145] Furthermore, the old observations of Huggins[146] and also those of Dr Gutman[147] (and I hope Dr Gutman will comment on this) indicated that the acid phosphatase of the serum in patients with carcinoma of the prostate is not elevated until the metastases and dissolution of bone occur

Now let us turn to the kidney Is the effect of parathyroid hormone on phosphorus excretion mediated through the alkaline or the acid phosphatase of the kidney? You will notice that the kidney increased in size, and the reason for this increase is that a very severe calcification of the kidney tubules occurred, accompanied by a marked decrease in the alkaline phosphatase Now which is the cart and which is the horse? Does the decrease in alkaline phosphatase occur first and deposition of the calcium salts follow, or is it *vice versa*? It should be noted that decreases in alkaline phosphatase occur under other conditions that cause disturbances in kidney function The most marked effect is obtained when hydronephrosis is produced by clamping off the ureters [148] When 'physiological' doses of parathyroid extract are administered to rats over a long period of time, no changes in the kidney phosphatases are observed, suggesting that the decrease observed with the high doses of parathyroid extract is secondary to calcification of the kidney tissue

Bartter Is the fall in alkaline phosphatase computed on a weight basis or on an absolute basis?

Kochakian It is on a total basis, that is total units

Acute Experiments with a Single Dose of Parathyroid Extract

The question might be raised that the 'physiological' doses of parathyroid extract were not sufficient to produce any metabolic changes in the phosphorus of the serum or of the urine Therefore, we decided to carry out exploratory experiments on the response of the rat to parathyroid hormone

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[145] Klendshoj N C and Koepf G F Serum Phosphatase Activity in Hyperparathyroidism, *J Clin Endocrinol* 3 351 352 (1943)

[146] Huggins, C and Hodges C V Studies on Prostatic Cancer I The Effect of Castration, Estrogen and Androgen Injection on Serum Phosphatase and Metastatic Carcinoma of the Prostate *Cancer Research* 1 293 297 (1941)

[147] Gutman, F B Sproul E E and Gutman A B Significance of Increased Phosphatase Activity of Bone at the Site of Osteoplastic Metastases Secondary to Carcinoma of the Prostate Gland *Am J Cancer* 28 485-495 (1936)

[148] a Wilmer H A Disappearance of Phosphatase from Hydronephrotic Kidney *J Exper Med* 78 225 230 (1943)
b Wilmer, H A Renal Phosphatase, Correlation Between Functional Activity of Renal Tubule and Its Phosphatase Content, *Arch Path* 37 227 237 (1944)

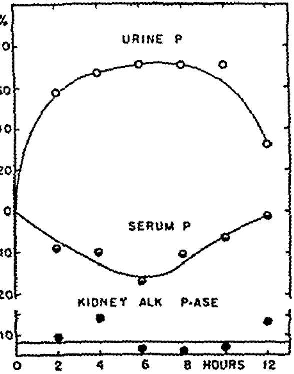


Fig 28 The Effect of a Single Injection of Parathyroid Extract on the Urinary Inorganic Phosphorus on the Serum Inorganic Phosphorus, and on the Kidney Alkaline Phosphatase Levels of Fasting Male Rats

The animals were fasted for 15 to 17 hours prior to the subcutaneous injection of 200 units of parathyroid extract (Lilly)

[*Reproduced by permission from Kochakian C D and Terepka A R Effect of* Parathyroid Extract on Tissue Enzymes and Serum and Urinary Phosphorus, *Am J Physiol* 165 142 148 (April 1951)]

It is commonly believed that rodents are very resistant to parathyroid extract We were pleased therefore to find that the rat did respond very nicely with both the serum phosphorus and the urinary phosphorus levels when relatively small doses of parathyroid extract were administered These effects are demonstrated by Figure 28 in which groups of experimental and control animals were killed at 2, 4, 8 10 and 12 hours after the administration of a single injection (200 units) of parathyroid extract You can see that the urinary phosphorus excretion increased to a peak at about 4 hours, where it remained for several hours, and by 12 hours it

had returned to approximately the normal level The serum phosphorus *on the other hand*, gave exactly the reverse picture, thus the rodent responds to parathyroid extract in a manner similar to the dog or the man[149] It also can be seen from this graph that the alkaline phosphatase of the kidney does not seem to be concerned with these two changes The values for this enzyme in the kidney remained constant throughout the entire experiment

Acute Experiments Done Elsewhere with Opposite Results

After we had finished these studies, Bonelli and Sala[150] of Italy reported an increase in the alkaline phosphatase of the kidney after administration of a single dose of parathyroid extract (Figure 29) The increase interestingly enough coincided with the time when we had observed a marked change in the serum and the urinary phosphorus levels *Although the total* change was 60 per cent, the results might have been fortuitous because the number of animals used was not more than two at each time interval We, however, did not wish to dismiss these experiments, for it was possible that some factor in the conditions of their experiments permitted the changes to appear Therefore, I wrote to Dr Bonelli, asked him for the complete details of his experiments, and suggested that we carry out simultaneous experiments in both laboratories using each other's methods for *determinations of the enzymes*, and using identical conditions in setting up the experiments I arranged with Eli Lilly and Company[142] to provide parathyroid extract to Dr Bonelli so that we could also use the same material for the injection

A comparison of the two procedures reveals the following differences in *the phosphatase determinations*

(a) The Italian investigators autolyzed the homogenate of kidney for 48 hours at room temperature We autolyzed ours for 24 hours at approximately 35° F (that is, in the refrigerator)

(b) The Italian workers used the Bodansky method and a pH of 8 6 We on the other hand used the King and Armstrong method and a pH of 9 7

Therefore, in repeating our experiments we measured the enzymes not only by the procedures that we had used before, but also by the procedures that

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149a Albright F, and Reifenstein E C Jr *The Parathyroid Glands and Metabolic Bone Disease Selected Studies* The Williams & Wilkins Company, Baltimore (1948)
b Shelling D H *The Parathyroids in Health and in Disease* C V Mosby Company, St Louis (1935)

150 Bonelli, M, and Sala G The Influence of Parathormone and of Vitamin D, on the Phosphatase Activity of the Kidney of the Rat, *Arch internat pharmacodyn* 80 457-463 (1949)

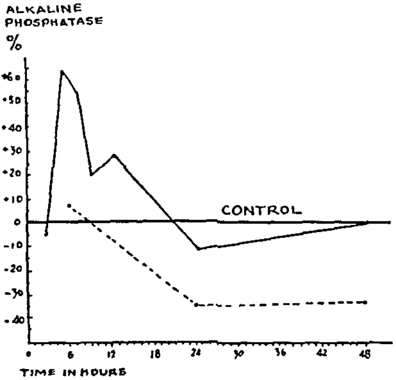


Fig 29 The Per Cent Variation in the Alkaline Phosphatase of the Kidney of the Rat Following Parathyroid Extract and Vitamin D_2

The heavy line at zero is the control line the *solid line* is the variation after parathyroid extract and the *broken line* is the variation after vitamin D_2

[Reproduced by permission from Bonelli M and Sala G The Influence of the Parathyroid Hormone and of Vitamin D_2 on the Alkaline Phosphatase of the Kidney of the Rat *Arch Int en Pharmaco et Therap* 80 457 (1949)]

had been used by the Italian investigators and furthermore we used both pHs in performing both determinations We repeated the experiments with an injection of 1 cc and also of 2 cc of the parathyroid extract In both instances we found that the parathyroid extract did not produce an *effect on the kidney phosphatase by any of the methods* As we expected the determinations at pH 8 6 by both the Bodansky and the King Armstrong methods were much lower than those at pH 9 7 Also the homogenates autolyzed at room temperature showed lower values than those autolyzed in the refrigerator

Since we had postulated in our publication that possibly some other phosphatases might be involved in the excretion of phosphorus after para thyroid extract treatment we determined the adenosinetriphosphatase ac tivity of the kidney but again we found no changes

All of these data have been sent to Dr Bonelli and we are eagerly awaiting the results of his experiments (In September 1952 Dr Bonelli reported that he and Dr Sala were not able to confirm their previous results in experiments duplicating their original studies and also in experiments identical with those of Dr Kochakian Dr Kochakian and Dr Bonelli agree that the divergent findings originally published were based on obser vations on an insufficient number of animals)

Conference Discussion

Armstrong You are probably right that the phosphaturia after para thyroid administration does not produce an increase in phosphatase activity in the kidney However I would like to ask you why you think an *in vitro* assay of phosphatase has any relation to the activity of phosphatase in the renal tubules?

Kochakian In other words you think that what I really should do is to canulate the afferent and efferent blood vessels of the kidney and then to determine the kidney phosphatase production by analyzing the enzyme content of the in coming and out going blood Is that the way I should interpret your question?

Armstrong The point is that the phosphatase activity is assayed by producing a situation in which one allows under laboratory conditions an extract of the tissue to hydrolyze a phosphoric acid ester The amount of phosphatase activity found under these artificial conditions may bear no relationship to the activity in the cell

Kochakian I think that is an objection which one can raise for all *in vitro* work on enzymes The fact still remains however, that you can alter the phosphatase in the kidney by several means I have already indicated that according to the literature damage to the kidney by constriction of the ureters[149] or by nephrotoxic agents[151] will produce a marked decrease

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[151]a Breedis C Flory C M and Furth J Alkaline Phosphatase Level in Urine in Relation to Renal Injury *Arch Path* 36 402-412 (1943)

b Hepler O E Gurley H and Simonds J P Experimental Nephropathies Renal Phosphatase After Poisoning With Mercury Bichloride Uranyl Nitrate and Potassium Dichromate *Arch Path* 39 133 141 (1945)

in alkaline phosphatase activity Furthermore androgens will produce an increase in this enzyme at low doses and a marked decrease at high doses [152] These are just examples

Armstrong Which way does androgen change the results

Kochakian It depends on the dose of the androgen First you get a small increase and then a very marked decrease not only on a unit per gram basis but also on a total basis

Fartter But Dr Kochakian on the basis of the available evidence that parathyroid works by decreasing phosphate transfer by the renal tubules wouldn t you expect perhaps a fall of phosphatase?

Kochakian That is what I was looking for at the time instead of the increase

Howard Does the serum acid phosphatase rise?

Kochakian We didn t do the serum acid phosphatase

Howard We have looked for changes in the serum acid phosphatase in hyperparathyroidism and never found any at all

Kochakian Well Koepf at Buffalo N Y has found an increase in the serum acid phosphatase in hyperparathyroidism [115]

Gutman We did some work on this problem a good many years ago In fact in our initial paper on acid phosphatase and alkaline phosphatase of prostatic tumor metastases in bone [117] we called attention to the presence of small amounts of acid phosphatase in bone extracts but because of the presence of large amounts of red cells and other extraneous material I could never be sure that the acid phosphatase present was derived from the bone and not from red cells or marrow tissue Subsequently we examined the serum for acid phosphatase in various bone conditions to see how specific the elevation in serum acid phosphatase was for metastasizing prostatic carcinoma We found that in Paget s disease with very extensive bone involvement and very high serum alkaline phosphatase that the acid phosphatase levels may also be elevated to some extent usually not above 6 or 8 units No correlation could be made between the alkaline and acid phosphatase levels however suggesting that when there is very extensive Paget involvement of bone some acid phosphatase derived from bone is liberated into the blood serum We subsequently found that in such situ

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[152] Kochakian C D Metabolic Influence of the Androgens *Progress in Clinical Endocrinology* edited by Soskin, S Grune & Stratton, New York (1950) pp 429 438

ations as multiple myeloma with very extensive skeletal destruction slight elevations in acid phosphatase might occur But the most convincing evi dence for the osseous origin of serum acid phosphatase under these circum stances was obtained in hyperparathyroidism with very extensive bone involvement in which the serum acid phosphatase level is as likely as not to be slightly elevated to 5 or 6 or 7 units When the parathyroid tumor was removed these serum acid phosphatase levels promptly fell to normal parallel with the serum calcium indicating I think that the serum acid phosphatase in hyperparathyroidism is derived from an osseous source The serum alkaline phosphatase is usually not significantly affected by the operation for many months

Sobel I would like to add one comment in connection with the presen tation namely in measuring increasing phosphatase values one factor is the amount of phosphatases the second the stage of activation I believe that the phosphatase activity operating on ATP should have been meas ured rather than as is usually the case the activity in terms of splitting glycero or phenylphosphates The results might have been entirely dif ferent

Kochakian We did determine the adenosinetriphosphatase and obtained no effect

Sobel ATP in the presence of phosphatase that is organic synthesis would have been the critical system to examine if we assume that excre- tion of phosphate is due to lack of organic phosphate formation and that resorption of phosphate is due to organic phosphate formation The general assumption is that reabsorption of glucose takes place by organic phosphate formation If organic phosphate formation does not take place then the system which produces the phosphatase is diminished in activity and the whole system as much as the enzymes is involved That is the explanation of phloridzin diabetes as I understand it the phloridzin interferes with organic phosphate formation In this explanation the possibility of pyro- phosphate synthesis is usually overlooked as an alternate explanation

Kochakian I am not ready to accept the phloridzin experiments to which you are referring I doubt that the conclusions derived from the experimental evidence are warranted I believe that Dr Gutman is of the same opinion

Follis As long as Dr Kochakian has put my name in the record I feel I have to say something We did not go into pathological bone destruction

concentrations of calcium and phosphorus are of tremendou[illegible] p[illegible] as far as bone destruction is concerned

It has been shown that if you give citrate to bind calcium bone is destroyed in order to maintain a normal serum calcium level The same is true probably, in acidosis We have been working on this using chelating agents and find the same mechanism It is quite possible that a similar situation in part exists in hyperparathyroidism When the concentrations of calcium and/or phosphorus are decreased in the serum bone is destroyed But what is going on in the normal state I don t know Perhaps Dr Howard has some comments on that

Howard I don t know whether you mean tho e studies on intravenous calcium or calcium deprivation Those were clinical observations

Follis Well what is the role of the humoral concentration which I think is important in bone destruction?

ations as multiple myeloma with very extensive skeletal destruction slight elevations in acid phosphatase might occur But the most convincing evidence for the osseous origin of serum acid phosphatase under these circumstances was obtained in hyperparathyroidism with very extensive bone involvement in which the serum acid phosphatase level is as likely as not to be slightly elevated to 5 or 6 or 7 units When the parathyroid tumor was removed these serum acid phosphatase levels promptly fell to normal parallel with the serum calcium indicating I think that the serum acid phosphatase in hyperparathyroidism is derived from an osseous source The serum alkaline phosphatase is usually not significantly affected by the operation for many months

Sobel I would like to add one comment in connection with the presentation namely in measuring increasing phosphatase values one factor is the amount of phosphatases the second the stage of activation I believe that the phosphatase activity operating on ATP should have been measured rather than as is usually the case the activity in terms of splitting glycero or phenylphosphates The results might have been entirely different

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Kochakian I am not ready to accept the phloridzin experiments to which you are referring I doubt that the conclusions derived from the experimental evidence are warranted I believe that Dr Gutman is of the same opinion

Follis As long as Dr Kochakian has put my name in the record I feel I have to say something We did not go into pathological bone destruction this morning partly for reasons of time and partly because we were trying to stick as much as possible to the normal situation I think the humoral concentrations of calcium and phosphorus are of tremendous importance as far as bone destruction is concerned

It has been shown that if you give citrate to bind calcium bone is destroyed in order to maintain a normal serum calcium level The same is true probably, in acidosis We have been working on this using chelating agents, and find the same mechanism It is quite possible that a similar situation in part exists in hyperparathyroidism When the concentrations of calcium and/or phosphorus are decreased in the serum, bone is destroyed But what is going on in the normal state I don t know Perhaps Dr Howard has some comments on that

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STUDIES ON THE RELATIONSHIP OF THE SERUM CALCIUM LEVEL TO PARATHYOID GLAND FUNCTION[153]

JOHN EAGER HOWARD

From the Department of Medicine The Johns Hopkins University School of Medicine and the Johns Hopkins Hospital Baltimore, Maryland

Howard We have certain suggestive data which allow of an hypothesis on certain features of parathyroid hormone function Dr Theda Hopkins working in our laboratory, found that approximately 70 per cent of the calcium of normal serum is ultrafilterable The technic used was that of Lavietes [154] by which the ultrafiltrate is derived through a duPont 300 cellophane membrane, anaerobically, with controlled pH (by saturating with 5% CO_2 in O_2) under 20 cm of mercury pressure When calcium salts were added to normal serum *in vitro* the percentage of ultrafilterable calcium remained 70 per cent of the total calcium and all the phosphorus was ultrafilterable *until* the total calcium reached a concentration of 20 mg per cent At this point and at points higher, there appeared a sharp falling off in filterable phosphorus and usually also in filterable calcium We interpreted this to mean that a colloidal complex had formed, involving calcium, phosphorus and protein There was no clouding and no sediment after centrifuging

The Effect of Intravenous Administration of Calcium on Normal Persons

To see whether the same rules held when hypercalcemia is produced *in vivo*, injections of calcium salts (gluconate or heptonate) were given to normal persons in quantities sufficient to raise the serum calcium from a normal level of 10, to 13 or 15 mg per cent Serum taken from these patients and subjected to ultrafiltration showed the same relationships between calcium and phosphorus and protein as had the sera to which calcium had been added *in vitro*

But coincident observations were quite interesting As the serum calcium rose by virtue of the injection of calcium salts, the serum phosphorus

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[153] Expenses of these studies were defrayed by a contract between the Johns Hopkins University and the Atomic Energy Commission

[154] Lavietes P H Anaerobic Ultrafiltration *J Biol Chem* 120 267 275 (1937)

rose also (as previously noted by Salvesen[155] in the dog and by Bassett[15] in the human) but the urinary phosphorus excretion fell despite no change in urine flow This seemed most remarkable and reminded one closely of the serum and the urinary phosphorus behavior in hypoparathyroidism Dr Hopkins suggested that the elevation of the serum calcium shuts off the release of parathyroid hormone to the circulation

The quantity of phosphorus required for the serum phosphorus to rise as much as it did was many fold greater than could be explained by the reduced renal phosphorus output So phosphorus must have come from cells We suspect therefore that parathyroid hormone alters the ratio between organic and inorganic phosphorus within cells and thus the serum phosphorus rises in hypoparathyroidism and falls in hyperparathyroidism

If we return to Dr Hendricks concept of calcium ions on the surface of many acres of tricalcium phosphate crystals in the bones these ions being available for support of serum calcium in times of need then such calcium ions must come off either easily or with difficulty depending on certain environmental factors One could conceive of a fall in pH as making them come off more easily a rise in pH less easily One could also conceive of a rise in inorganic phosphorus making them come off *less* easily, a fall in PO_4 concentration *more* easily Thus the parathyroid hormone by lowering the serum phosphorus would enhance the availability of skeletal calcium hypoparathyroidism by elevating the serum phosphorus would reduce the availability of skeletal calcium Thus the parathyroid glands would act as a sort of calciostat for the serum, its action carried out *via* the bones

The Effect of the Intravenous Administration of Calcium to Patients with Parathyroid Disease

According to these theories a patient with a parathyroid adenoma should not behave like a normal person to artificial elevation of serum calcium for the elevated serum calcium could not shut off the parathyroid activity of the adenoma as it does that of the normal gland In support of this thought we found that four patients with subsequently proved parathyroid adenomas have responded to intravenous calcium injection in a different man

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[155] *Salvesen H A Hastings A B and McIntosh J F The Effect of the Administration* of Calcium Salts on the Inorganic Composition of the Blood *J Biol Chem* 60 327 339 (1924)

[156] Bayler C H Van Alstine H E Keutmann F H and Bassett S H The Fate of Intravenously Administered Calcium Effect on Urinary Calcium and Phosphorus Fecal Calcium and Calcium Phosphorus Balance *J Clin Investigation* 29 1167 1176 (1950)

ner from the normal person The serum phosphorus of these patients rose little or none and the urinary phosphorus increased rather than decreased

Two hypoparathyroid patients (in whom one expects little or no parathyroid tissue capable of responding to stimuli) likewise responded to intravenous calcium injection in a different manner from normal subjects In these patients the serum phosphorus rose little or none and the urinary phosphorus rose

These observations seemed worthy of consideration though of course they do not prove the theories presented I wanted to ask our chemist friends here if the thoughts presented about phosphate ion increases making calcium harder to remove from bone crystal surfaces and *vice versa* make any chemical sense

Conference Discussion

Edelman I would like to ask you a question Dr Howard In order to make the calculation that the phosphate did not come from conservation of urinary phosphate you had to assume a phosphate volume of distribution What volume did you assume?

Howard I assumed no intracellular phosphate change I grant you

Edelman That would make a difference wouldn t it?

Howard Well it would make the difference on my side

Edelman Yes in other words assuming a larger volume of distribution would require more phosphate

Howard I would have to put another 100 on it

Edelman Right The other point I wanted to raise was that Kurt Lange has said that injecting calcium salts in patients with nephrosis produces a fall in the filtration rate

Howard In filtration rate?

Edelman Yes in the glomerular filtration rate It has been observed that an elevation of the serum calcium in patients with destructive bone disease frequently will produce a uremia which is reversible when the serum calcium is lowered [157,158] I wonder whether there might be a direct action

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[157] Herrmann J B Kirsten F and Krakauer J S Hypercalcemic Syndrome Associated with Androgenic and Estrogenic Therapy *J Clin Endocrinol* 9 1 12 (1949)

[158] Swyer A J Ber[illegible] Gordon, H M and Laszlo D Hypercalcemia in Osteolytic Metast[illegible] *[illegible]ed* 8 724 732 (1950)

on the kidney which might account at least in part for the changing pat terns of excretion

Howard Of urinary phosphorus excretion?

Edelman Yes

Howard Well I think that is a criticism that is perfectly valid Dr Neuman has not done for us yet even on the normal individual inulin clearances coincident with this intravenous calcium test We did not want to do this because inulin is bulky stuff and I don t know what it might do to water relationships and whatnot But I must confess that it seems un likely to me that calcium reduces the filtration rate We gave somewhere between 700 cc and a liter of fluid in the period of three hours We judged from the hematocrit and serum protein concentrations how much dilution we got in the extracellular compartment which is crude I admit but that again favored the interpretation I am talking about and was taken into consideration

Edelman Yes I am not questioning your hypothesis as to the source of the additional phosphate in the serum

Howard But the urine volume and the urine nitrogen remained fairly constant so I think that the filtration rate anyway was not lowered It may have been a little but certainly not to a degree to account for the fall in phosphorus excretion

Copp I would like to say something in support of Dr Follis humoral theory We found that when normal rats were put on a low phosphorus diet within 24 hours there was a very marked increase in calcium excretion which occurred long before there were any histological changes and with out any marked fall in the level of serum calcium The only biochemical change observed was a slight fall in the serum inorganic phosphorus I think this and the data which Dr Hastings presented at the last meeting[159] showing the difficulty in raising the serum calcium by infusion of intra venous calcium both indicate that there is a tremendous capacity of bone to give up or to take up mineral This may not actually represent bone destruc tion in the early stages It may possibly be an effect on calcium ions on the surface of the bone crystals as Dr Hendricks could judge better than I but certainly it occurs long before you get any histological evidence of osteoblast activity or bone resorption

Follis That is the trouble, of course in the normal state, as Dr Hen

[159]Hastings A B Studies on the Effect of Alteration in the Concentration of Calcium in Circulating Fluids on the Mobilization of Calcium *Trans Macy Conference on Metabolic Interrelations* 3 38 50 (1951)

dricks pointed out last time there is this store of calcium adsorbed on the crystal so that *you have to employ very drastic measures* to use up that store before you can begin to draw on the bone crystal itself and we assume that only then you will destroy the matrix as well I think therefore you have to expose the individual to very drastic treatment before you get these pathological forms of bone destruction

Copp Perhaps the best examples are Dr McLean's pigeons (or were they chickens?) during the egg laying stage

Neuman One point bothers me a great deal I can t help feeling that the extracellular fluids are *exactly* saturated with respect to the inorganic components of bone because a few liters of extracellular fluid are flowing over two acres of mineral surfaces and spread out as a film that is an *extremely thin layer It seems impossible that the fluids are not exactly in* equilibrium with the surfaces of all those crystals Yet it is a pretty well established physiological fact that the ion products can vary all over the place When one hears of a case in which the serum calcium level rises to 17 mg % accompanied by a rise in the serum phosphate level but with no change in the bicarbonate level one is hard put to find a logical explanation There is something very inadequate about our knowledge of that equilibrium

Fremont Smith Does it help at all to know that the spinal fluid calcium *does not go up when the blood calcium goes up?*

Copp I should like to know also the concentration of the diffusible *calcium ion*

Howard It holds exactly Dr Hastings doesn t like the way I express *this relationship and it is possible to define as he did for us when we gave* him these figures the pK for calcium protein But to me a simpler way of visualizing the relationship is this when the calcium of the serum in the ultrafilter is 10 the calcium of the ultrafiltrate is approximately 7 provided the pH is maintained Now pH changes vary the results markedly The data I have reported were *all obtained at pH values from 7 3 to 7 45 When we added calcium and increased the total calcium* to 17 70 per cent was still found in the ultrafiltrate The protein binds more under these circumstances or however you want to express it That same relationship held true in the serum that we took from the normal subjects after we had artificially raised their serum *in vivo* to about the same *high levels*

However when we reached a level of 20 for the calcium *in vitro* and then used the ultrafilter there was a very sharp and *distinct point in* the presence of a normal serum protein a normal phosphorus and a calcium of 20 at which there was a marked falling off of the ultrafiltrate content of

calcium It was no longer 70 per cent and the phosphorus which had been 100 per cent diffusible, fell off too, they both decreased to approximately 30 to 50 per cent Therefore a point is reached where a colloidal complex of some sort is formed which does not pass the filter

Copp I have a question for the biochemists If there is a colloidal precipitate of calcium phosphate which accounts for the excess that is found in the serum when the serum is dialyzed would this precipitate dissolve and dissociate as the calcium and phosphorus ions diffuse into the dialyzing fluid?

Neuman No

Howard It does not precipitate, that is all I know I don t know what the state of the calcium phosphorus protein complex is

Copp It doesn't have to be precipitated as long as it is not in ionic form

Neuman May I express my ignorance here and ask if there is any clinical situation in which both the calcium and the phosphate of the serum go down?

Armstrong Yes we see this situation in rickets

Howard Yes There are two conditions in which both the calcium and the phosphate of the serum are low The first of these is the situation that follows the removal of a parathyroid tumor The serum calcium level falls below normal while the serum phosphorus level doesn t rise to normal for a long time after the tumor is removed in most cases, it stays low for months The other situation in which we find both minerals low is in osteomalacia or rickets, as in steatorrhea where the serum calcium may be 6 and the phosphorus is usually 4 or 5 Now, such a patient is usually in tetany, and if you correct the tetany by giving him a high calcium intake you precipitate most of the phosphorus in the diet and make the serum phosphorus level fall and still the serum calcium is only about 7, you have barely raised it You can bring the phosphorus level down to 1, so that no phosphorus appears in the urine at all for months on end

These, therefore Dr Neuman are the two circumstances in which the situation that you asked about occurs I wonder if the available ions on the surface of these acres that you are talking about, the surface calcium ions are influenced by two factors, one of which is the pH, with alkalinity tending to make it harder to bring them off and acidity tending to make it easier to bring them off and the other the level of serum phosphorus, with a high chemical pressure of phosphate ions tending to make it more difficult to bring them off and a low pressure tending to make it easier to bring

them off In other words the bone would support the serum calcium when the phosphorus was 6 or 8 only if the serum calcium fell to 8 instead of staying at 10 Do you see what I mean? Well that is the hypothesis and I don t know whether or not it is chemically sound

Hastings Dr McLean wants to answer you

Howard Good!

McLean First I might say that the natural history of colloidal calcium phosphate has been pretty thoroughly worked out in the living animal If phosphate is injected intravenously into the dog and the phosphate concentration in the plasma is raised the calcium will drop We have shown both by studies of calcium ion concentrations in the blood under such circumstances [160] and by the demonstration of Gersh[161,162] microscopically that colloidal calcium phosphate although it is not precipitated out of the plasma is picked up by the macrophages and can be identified in them It disappears very quickly and apparently is redissolved returned to the circulation and then excreted Whenever the ion product that represents saturation with both calcium and phosphorus is exceeded colloidal calcium phosphate is formed McLean and Leiter[163] published a paper some years ago about a similar finding in cases of uremia with low calcium and tetany *in which apparently colloidal phosphate is formed and removed from the* blood

There is one remark that has been going around the table that I would like to correct that is about the relation of pH to possible calcification There has been in the literature and it has been referred to several times today an idea that raising the pH promotes the deposition of calcium phosphate and hence calcification On theoretical grounds this is not very sound for the reason that at the pH of the blood the ion product is not increased very much by increased alkalinity There is some change with an increase in the tertiary phosphate ion and none virtually none in the secondary phosphate ion Perhaps even more convincing than that and I think Dr Sobel can bear this out if one deliberately takes a sample of plasma or serum which will not itself cause calcification of rat slices *in vitro* and shakes out the CO_2 to the point of a very marked increase in pH the

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160 McLean F C and Hinrichs M A The Formation and Behavior of Colloidal Calcium Phosphate in the Blood *Am J Physiol* 121 580-588 (1938)

161 Gersh I Histochemical Studies on the Fate of Colloidal Calcium Phosphate in the Rat *Anat Rec* 70 331 349 (1938)

162 Gersh I The Fate of Colloidal Calcium Phosphate in the Dog *Am J Physiol* 121 589 594 (1938)

163 McLean, F C and Leiter L The State of Calcium in the Blood in Nephritis and Uremia *J Clin Investigation* 14 705 (1935)

ability of the serum to calcify rat slices will not be changed You have observed this many times Dr Sobel I am sure as have we

Sobel Yes I have observed this although not as convincingly as you have

McLean Well have you ever seen a solution that would not calcify a rat slice be altered so that it would calcify the slice by a change in its pH?

Sobel No It is possible that this can take place but I have never observed it From my observation as one either increases or decreases the pH of a given solution there appears to be less calcification *in vitro*

McLean Well let me state this and this also refers to the question of equilibrium No one has yet accounted for the fact that the infant s plasma phosphate is twice as high as the adult s by simple chemical analysis Also it is very easy to demonstrate that the serum of a normal infant will calcify rachitic slices That goes clear back to Holt

Follis It is Shipley [164] Dr McLean not Holt

McLean I m sorry yes The serum of the adult with its lower phosphate will not calcify the rachitic slices If enough phosphate is added to that serum to bring up the level to the level of the infant s plasma or serum then it will calcify slices *in vitro* It will not do so if the pH is increased simply by shaking out the carbon dioxide which will bring it up well above 8

In view of these findings it seems first very unlikely that any alkaline change in pH compatible with life has anything whatever to do with the deposition of calcium phosphate Then with reference to the differences between infants and adults if we say that the serum or plasma of a human being is in equilibrium with the bone salt are we speaking of the infant state or of the adult state? I don t know I am asking for information

Hendricks Is the calcium under those conditions the same?

McLean It is the same as I have noted it

Shorr Would it have any bearing on your thinking if the blood citric acid in hyperparathyroidism were to rise a matter of 1 5 to 2 5 mg per cent with the rise in serum calcium? Because that is what does happen

McLean We followed the calcium ion by direct observation in cats following injection of parathyroid extract and following parathyroidectomy We followed the blood calcium all the way down and all the way up in the

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[164] Shipley P G The Healing of Rickety Bones *In Vitro* *Bull Johns Hopkins Hosp* 35 304 (1924)

same animals and we were able to show that in every instance the mass law relationship between calcium and protein was at least approximated It is not a very accurate method but there was no indication that either in hyper or in hypoparathyroidism was there any introduction of a new calcium binding substance into the plasma

Shorr Our studies in human hyperparathyroidism do show this parallelism between the levels of blood calcium and blood citric acid After removal of a parathyroid tumor and a fall in the serum calcium values there is a corresponding fall in the blood citric acid level Whether or not these changes in citric acid are of sufficient magnitude to influence the state of calcium in blood is another matter on which I should like to have some comment

McLean The one instance in which the citric acid level apparently is important is in the rabbit The rabbit has a considerable quantity of citric acid in the plasma particularly on certain diets that lead to citric acid formation and the serum calcium in the rabbit will go up and down parallel with the citrate in the presence of a constant protein concentration

Howard May I answer one comment of Dr McLean s on this colloidal precipitation business which was directed to us? He referred to the experiments in which Gersh injected a strong phosphate solution into animals and found that the clasmatocytes were taking up calcium phosphate The solution that he used must have raised the serum phosphorus to the neighborhood of 40 mg which is away out of proportion to anything that I am talking about here

We tried to do the same type of intravenous experiment with phosphorus instead of with calcium to see what would happen to the serum calcium level The serum calcium did not fall when we injected neutral phosphate salts into the serum and raised the serum phosphorus level from 3 5 to 8 The serum calcium fell only to the point at which we had diluted the serum as judged by the plasma protein the red count and the hematocrit The calcium did not fall below this point and the diffusibility or ultrafilterability of it was exactly the same as predicted from the pK Furthermore the phosphorus was 105 per cent diffusible

Bartter How long did you carry out that infusion?

Howard Over a four hour period

Bartter May I ask Dr McLean a question? Do you know whether or not all of the serum phosphorus in the child is diffusible [165]

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[165] McCance R A Renal Physiology in Infancy *Am J Med* 9 229 241 (1950)

Howard We have done this a great many times and the answer is "Yes"—all but a very insignificant portion is ultrafilterable

Hastings May I say a word to Dr Shorr about the citrate? Two milligrams per cent would be about a tenth of a millimole per liter, which would account for at most only about 5/100ths of a millimole of calcium per liter, so the citrate would have a negligible effect

Howard I have just one other comment Dr McLean pointed out that the serum of a normal human infant contains twice as much phosphate as that of a normal human adult and therefore that the serum of the infant will calcify rachitic cartilage *in vitro* while that of the adult will not The obvious point is that the rachitic cartilage to which he refers is *the rachitic cartilage of the rat* an animal which (as a normal adult) has a serum phosphorus level around 6 or 7 the same as that of a normal human infant *The rachitic cartilage of the human* might calcify perfectly well with serum of a normal human adult

Kochakian I think the rat serum phosphorus level is about 10 mg per cent

Shorr What happens to the equilibrium between calcium and protein in the course of an ultrafiltration as the filterable calcium passes through the membrane?

Howard Well, that is an interesting problem You would think that the dynamic equilibrium would make a change from hour to hour Actually we are using a pressure of 25 centimeters of mercury, which is higher than any capillary pressure, because we can t get enough across the membrane otherwise But if you will grant me this difference between our system and the capillary membrane, the interesting point is that when you get 1 cc from your 12 at the end of four hours, you get exactly the same concentration of calcium in it, as well as in the residue as when you get 6 cc at the end of 48 or 72 hours, in other words the relationship does not change Now we are using a membrane which sits at an angle under mercury pressure so that the surface between the filtrate and the residue is never more than momentary It runs right off to the top of the tube, you see, and the whole procedure is done anaerobically This may explain it But it is interesting that you can account for 100 per cent of the initial calcium content by the sum of the calcium in the residue and the calcium in the ultrafiltrate, and that the same concentration relative to water is maintained at one hour as it is at 72 hours We thought this meant at least that the relationship was more or less good and sound and fixed

Reifenstein I would like to ask Dr McLean how he fits into the picture

the effect of growth hormone upon the serum inorganic phosphorus level[166] The child who is growing presumably has a lot of growth hormone and his phosphorus level is high I have always thought that it was higher in the child than in the adult because of an increased amount of growth hormone In our acromegalics, we find about 1 mg per cent or 2 mg per cent higher values for the serum inorganic phosphorus level than in normal individuals provided gonadal activity is absent, I mean if the patients are menopausal or have had sufficient destruction of the rest of the pituitary gland so that the gonadotropin excretion is low As I remember it, Dr C H Li[167] showed that you obtain a marked fall in the serum inorganic phosphorus level when you hypophysectomize rats, and that you can restore the level in such hypophysectomized animals at least to normal by giving the Li growth hormone preparation Don t you think that a contribution to the inorganic phosphorus level from the growth hormone has to be considered at least when you are dealing with children?

McLean I don t know what the regulatory mechanism is for the serum phosphorus level

Follis It is quite likely also that the child is taking in more phosphorus isn t it? Dietary supplies may be higher Do you think you can elevate your serum inorganic phosphorus level by taking in more phosphorus?

Bartter Surely

Armstrong No, not really! Not from 4 to 6 mg per 100 cc

Bartter I think so If you take in more than your glomeruli can filter (and if phosphorus is not secreted as is generally agreed) then the serum phosphorus must rise

Copp In connection with the growth hormone, Dr Reifenstein, my un

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166a Albright F Reifenstein E C Jr and Forbes A P Effect of Estrogen on the Growth Hormone *Trans Conference on Metabolic Aspects of Convalescence* 12 97 104 (Feb 1946)

b Reifenstein E C Jr, Kinsell L W and Albright F Observations on the Use of the Serum Phosphorus Level as an Index of Pituitary Growth Hormone Activity The Effect of Estrogen Therapy in Acromegaly *J Clin Endocrinol* 6 470 (1946)

c Reifenstein E C Jr Kinsell L W and Albright F The Effect of Estrogen Therapy in Acromegaly with Observations on the Use of the Serum Phosphorus Level as an Index of Pituitary Growth Hormone Activity *J Clin Investigation* 25 932 (1946)

d Albright F Reifenstein E C Jr Forbes A P and Bartter F C Effect of Estrogens in Acromegaly, *Trans Conference on Metabolic Aspects of Convalescence* 14 102 122 (1946)

167 Li C H and Evans H M The Biochemistry of Pituitary Growth Hormone *Recent Progress in Hormone Research* 3 25 27 edited by Pincus G, Academic Press, Inc, New York, 1948

derstanding is that the growth hormone does not become active in the human until perhaps ten or eleven years of age and that there is very little growth hormone in the infant and the small child with their elevated serum phosphorus levels

Follis How does he grow?

Copp He just grows The growth hormone induced spurt in the human is a result of the increased activity of the pituitary during adolescence

Follis How do you account for hypopituitary dwarfs?

Meyer You can t make them grow with growth hormone either

Follis You can t make the human but you can the rat

Meyer Well you asked about the growth of the infant not the rat

Copp Even in the rat there actually is very little effect of growth hormone during the early stages of growth

Follis Suppose you take out his hypophysis?

Copp There is no effect if it is removed during the post natal period from 0 to 28 days which corresponds to the infant period During the next period in the rat s life which corresponds to adolescence growth hormone is active In the human these periods are separated in time In the hypopituitary dwarf of course you have a depressed thyroid activity and other features that go with this condition and which might depress growth

Reifenstein It is our concept Dr Copp that children have adequate amounts of anterior pituitary growth hormone almost from birth The spurt in growth you mentioned which occurs about the time of puberty is due we think to the effect of the increased amounts of hormones from the gonads and of gonadal like hormones from the adrenal cortex which are produced as part of the process of adolescence It is our belief that the growth hormone is present throughout life and is responsible in part for the higher serum inorganic phosphorus values of children and of old persons who have reduced gonadal hormone production The gonadal and gonadal like hormones by an undefined mechanism bring about some reduction in the serum inorganic phosphorus levels to produce the values we recognize as our normal adult levels [166]

Armstrong Is there any information as to possible differences in renal function of an infant and an adult? I am thinking of possible differences in renal clearance of phosphate between humans of different ages

Bartter Only in young infancy (under 1 year) is the filtration rate in the normal low enough to produce an elevation of serum phosphorus on an average phosphorus intake

Dziewiatkowski I am surprised that the question of the level of serum phosphatase hasn't been brought up Isn't it higher in the infant than it is in the adult? Wouldn't this be a possible explanation for the higher serum phosphate level in the infant?

Follis The bones are more active

Howard Serum phosphorus and phosphatase certainly do not parallel one another in the adult The serum phosphatase is presumably a spill over from tissue phosphatase, with liver disease or activity of new bone growth That is what is supposed to give you the alkaline phosphatase in the serum You can have very high phosphate level in the adult, and the phosphatase be very low

Neuman The problem we started with here was the matter of having a whole series of levels of serum calcium and serum phosphate which bore no relation to any kind of an ion product Then we introduced hormones It seems to me that we are getting into the problem of solubility, and I am surprised that Dr Ericsson has not said anything throughout this discussion If he won't I will

Ericsson I feel I should wait until tomorrow

Neuman Perhaps we should defer it until tomorrow However, if I understand the gist of your data, the actual serum levels of calcium and phosphate are much greater than predicted by such solubility measurements of the calcium phosphate system as have been made *in vitro* Dr Hastings, at dinner, was quite adamant that calcium carbonate does not account for the solubility We have a situation where the calcium phosphate system—whether or not calcium carbonate is present—will not account for the serum level Well, this brings me around to thinking as we have already said—I know Dr Hendricks stated it last year—that the requirements of the surfaces of these mineral crystals are much less rigorous than the requirements of the apatite or the carbonate apatite or any other type of structure we might pose In the bone mineral surface, the relative calcium concentration, the hydrogen ion concentration the other cation concentrations the phosphate, and carbonate substitutions, all may change This represents a relatively large mineral storehouse which can be altered in composition a great deal and can, perhaps, account for some of the variability observed *in vivo* It also follows that you cannot change serum levels short of really drastic means It should require time I don't think any of the data cited so far have contradicted this idea It takes four hours or so before these changes in phosphate concentration occur, and they take place only after you have deposited large quantities of calcium All of these changes in serum ion concentration involve rather large ion movements,

large ion deposition or large ion removal We have over and above the basic solubility a phenomenon that is quite unrelated to a solubility product but represents a quite variable structure that of the surface of this ionic lattice that we designate as apatite or alpha tricalcium phosphate or by some other name What do you think of such a view?

Sobel First of all the changes at least in our experience in rabbits take place much faster than that that is the intravenous injection of phosphate will depress the serum calcium and the intravenous injection of calcium will depress the serum phosphate within an hour This we studied in connection with lead poisoning Secondly I think that there are solubility factors in the serum For example female birds like the hen during the egg laying cycle can build up serum calcium to very high levels presumably due to a special protein that is present in the blood The potential for the formation of un ionized complexes is one of the determining factors for the total amount of calcium and phosphate in serum not only for the ionic product but for the total amount of calcium and phosphate that can be present, namely the basic solubility in the serum Of course the ion product in equilibrium with the bone will probably be the ultimate limiting factor but we have the kidney as an auxiliary factor at the same time

SOME RECENT FACTS ABOUT THE PROPERTIES OF TRICALCIUM PHOSPHATE AND THE COMPOSITION OF THE BONE SALT

MARCEL J DALLEMAGNE

From the Department of Pharmacodynamics Institute of Experimental Therapeutics School of Medicine University of Liege, Liege Belgium

Armstrong In the previous sessions we dealt with the soft tissues of bone Now we will deal with bone mineral which is a firmer subject (That was supposed to be a joke) (Laughter) We have, collected in this room some of the world s authorities on the composition of the mineral substance of calcified tissues At least three, and perhaps four divergent interpretations are made with respect to this subject I am sure that before the day is ended we will all be familiar with the different opinions and different interpretations and with the reasons for each opinion

About a year ago Dr Fremont Smith wrote to me to say that I might invite a guest from abroad to attend this meeting I have had several difficult decisions to make in my life but this was not one Who else could be invited to be the first foreign visitor to this Conference but Professor Dallemagne? I was very happy that he was able to arrange his schedule so as to come at this time of the year which is in the midst of his academic program Professor Dallemagne is Professor of Pharmacology in the Institute of Experimental Therapeutics of the University of Liege He has several research interests He works not only in the field of bone, as all of you are aware but also in pharmacology with special reference to drugs which affect neuromuscular transmission He will during his tour through this country lecture on that subject on several occasions as well as on the subject of the composition and the chemistry of bone

Professor Dallemagne will introduce the subject of bone mineral composition following which we will have a brief question period I think the conference will move most expeditiously if Dr Duncan McConnell then makes a brief presentation After this we can have a general discussion of the whole subject Professor Dallemagne will you please proceed?

Dallemagne Please excuse my poor English, as I seldom have an opportunity to speak your language in Europe I hope you will understand me First of all I want to extend to Dr Fremont Smith and to Dr Armstrong my most heartfelt thanks for all the kindness they have shown me in providing me with the occasion to come to the United States and to discuss with you the topic that is so interesting to all of us

It is not possible to deal with the chemical composition of bone salts without first discussing the properties of the calcium phosphates and particularly those of tricalcium phosphate

The Existence of Tricalcium Phosphate

As you know, the nature of tricalcium phosphate has often been debated during the recent years and even the very existence of this salt as a crystalline entity has been questioned In my opinion all of the uncertainty concerning the nature of this compound derives directly from the properties of tricalcium phosphate its crystalline particles are so small that they are able to adsorb many other elements particularly the reagents from which the salt is prepared in an aqueous medium The crystalline character of tricalcium phosphate is unquestionable as the substance diffracts x rays However the interference lines recorded are very diffuse which is a consequence of the smallness of the crystallites

Another factor which disturbs the interpretation is the problem of definition This is particularly the case for hydroxylapatite This name is sometimes applied not only to the true natural compound but also to tricalcium phosphate which has adsorbed calcium hydroxide in about the same proportion as in the molecule of hydroxylapatite

THE PROPERTIES OF HYDROXYLAPATITE

Hydroxylapatite is a complex salt composed of tricalcium phosphate and calcium hydroxide the relative proportions of which are indicated by the formula

$$[Ca_3(PO_4)_2]_3 \bullet Ca(OH)_2 \quad \text{or} \quad Ca_{10}(PO_4)_6 \bullet (OH)_2$$

that is to say three molecules of tricalcium phosphate for one of calcium hydroxide This substance exists naturally Its properties have been reviewed by Mitchell Faust Hendricks and Reynolds[168] It gives the same radiogram as tricalcium phosphate but the interference lines are fine which indicates good crystallization. [illegible]
temperature sor [illegible]
per cent of the [illegible]
high (between 1 [illegible]

Synthetic tricalcium phosphate and hydroxylapatite have often been mistaken for one another, because of the occasional similarities in the chemical composition of these substances

OPINIONS CONCERNING THE EXISTENCE OF TRICALCIUM PHOSPHATE

There are two different opinions about synthetic tricalcium phosphate and these can be summarized in a very simple way The basis of the dis

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[168] Mitchell L Faust G T Hendricks S B and Reynolds D S The Mineralogy and Genesis of Hydroxylapatite *Amer Mineral* 28 356 (1943)

cussion is the following fact when phosphoric acid and calcium hydroxide are mixed together with water, in such amounts as to correspond to the Ca/P ratio of tricalcium phosphate (1 94) the precipitate obtained has a Ca/P ratio which, according to circumstances, may vary between 1 94 and 2 14 or even more Greenwald[169] has found very high values (up to 2 79) but most commonly the ratio varies between 1 94 and 2 25

The First Opinion

According to some authors, tricalcium phosphate does not exist as a chemical entity In their opinion, the precipitate with a Ca/P ratio of 2 14 is pure hydroxylapatite When the value of the ratio is lower (down to 1 94) the substance would be hydroxylapatite having adsorbed some PO_4 ions A Ca/P *ratio higher than 2 14 would be due to the adsorption of* some calcium hydroxide This opinion has been supported by Klement[170] as well as by Hodge and collaborators[171]

The Second Opinion

However, according to other investigators tricalcium phosphate actually exists A precipitate with a Ca/P ratio of 1 94 would be pure tricalcium phosphat When the ratio is higher, there would be adsorption of some calcium hydroxide The important point is that this calcium hydroxide would not be fixed in the crystalline lattice unless the precipitate has been brought to a high temperature These two opinions are thus opposed to each other and we have supported the second one for quite a few years[17, 173]

ARGUMENTS FOR THE FIRST OPINION

The arguments which have been presented in favor of the first opinion are the following ones

Point 1

Radiograms of synthetic tricalcium phosphate and natural hydroxylapatite are identical[174]

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[169]Greenwald I Anomalous Effects in the Titration of Phosphoric Acid with Cal c m Hydroxide *J Am Chem Soc* 66 1305 1306 (1944)

Nat

tion 156

161 (1938)

[172]Dallemagne M J and Brasseur H The Diffraction of X rays by the Mineral Substance of Bone *Bull Soc Roy Sci Liege* 11 451 and 488 (1942)

[173]Dallemagne M J *The Chemical Nature of the Mineral Substance of Bone* Thesis for Appointment of Higher Teaching Gordinne Liege (1943)

[174]Taylor N W and Sheard C Microscopic and X ray Investigations on the Calcification of Tissue *J Biol Chem* 81 479-493 (1929)

Point 2

If tricalcium phosphate with a Ca/P ratio close to 2 14 is progressively heated up to 900° C, the radiogram does not change [175]

Point 3

The analysis of the precipitate only occasionally gives results corresponding to the theoretical composition of tricalcium phosphate

ARGUMENTS FOR THE SECOND OPINION

Our arguments in favor of the second opinion can be summarized as ollows

Point 1

Mineral hydroxylapatite is thermostable up to a high temperature whereas pre cipitated tricalcium phosphate loses water from 100 to 700° C until complete dehydration has occurred The amount of water released is variable but 5 per [illegible] has been brought [illegible] iginal substance [illegible] This fact had

From the composition of apatites in general, we have come to the conclusion that tricalcium phosphate contains two molecules of water for every nine atoms of calcium. This would amount to 3 87 per cent of water Such a composition [illegible]

$$[Ca_3(PO_4)_2]_3 \bullet Ca(OH) \quad \text{and}$$
$$[Ca_3(PO_4)_2]_3 \bullet H_2(OH)_2$$

The above formulas refer to the alpha form of tricalcium phosphate The beta form $Ca_3(PO_4)_2$ corresponds to the substance completely dehydrated at 700° C A somewhat different hypothesis has been proposed by Hendricks and Hill [177]

[illegible]

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[175]Tromel G and Moller H The Formation of Poorly Soluble Calcium Phosphates from Water Solution and the Relationship of These Phosphates to the Apatite Group *Z anorg allgem Chem* **206** 227 240 (1932)

[176]Schleede A Schmidt W, and Kindt H Contribution to the Subject of Calcium Phosphate and Apatite *Z Elektrochem* **38** 633 641 (1932)

[177]Hendricks S B and Hill W L The Inorganic Constitution of Bone *Science* **96** 255 257 (1942)

[178]Hendricks S B and Hill W L The Nature of Bone and Phosphate Rock *Proc Nat Acad Sc* **36** 731 737 (1950)

[179]Chaudron G and Wallaeys R Syntheses of Apatites by Solid Phase Reactions *Bull soc chim France* **16** 132 (1949)

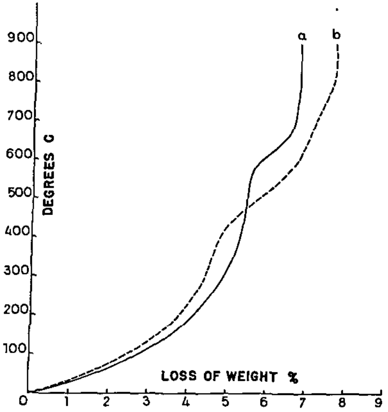


Fig 30 Dehydration Curves of Alpha Tricalcium Phosphate Prepared by Two Methods

Curve a was obtained by heating the same sample of synthetic alpha tricalcium phosphate progressively, and weighing it every 50 to 100° C. *Curve b* was obtained by heating each of a number of different samples of equal weight at a fixed temperature for 12 to 24 hours, and then weighing it The series of fixed temperatures was chosen in such a way as to give a reading every 50 to 100° C

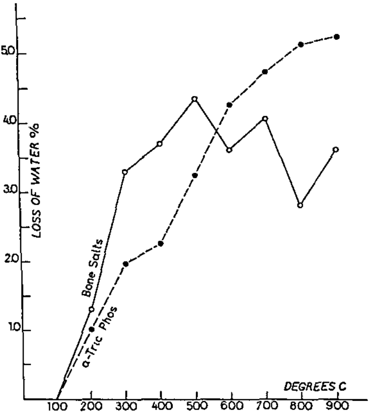


Fig 31 The Dehydration Curves of Synthetic Alpha Tricalcium Phosphate and of Bone Salts

The curve for synthetic alpha tricalcium phosphate is plotted from the same data as *Curve b* of Figure 30 *The dehydration curve of the bone salts* was obtained by the same method as was that of synthetic alpha tricalcium phosphate (see *Curve b* Figure 30)

the H O which is part of the crystalline lattice and this added water is also eliminated between 100° and 600° C As we said before the amount of water which is eliminated between 600° and 700° C is much more constant The precipitate partially dehydrated at 600° C is able spontaneously to recover the water which it has lost The completely dry substance obtained at 700° C is stable [179]

A typical dehydration curve of synthetic alpha tricalcium phosphate is shown in Figure 30a it can be obtained by using Chevenard s thermoscale or simply by progressively heating up a sample of the precipitate, which is weighed every 50° or 100° C However another method can be used instead of bringing one sample of substance through the whole range of temperatures one may use different

phosphate and hydroxylapatite

Point 2

The refractive index of alpha tricalcium phosphate is different from that of hydroxylapatite it is about 1 590 [180] while that of hydroxylapatite is higher (between 1 640 and 1 651)

Point 3

C in the presence
xide one obtains
nd the refractive

[180]Dallemagne, M J and Brasseur H The Nature of the Principal Salt of Bone Studied by X ray Diffraction *Experentia* 3 469-471 (1947)

[181]Wallaeys R and Chaudron
the Hydroxyl Radical for Ch
Fluorine *Compt rend Acad*

[182]Dallemagne M J Bone and the Mechanisms of its Formation Calcium Phosphates, Biochemistry of Ossification and Composition of Bone *J Physiologie* 43 425 (1951)

Point 4

[illegible]

tricalcium phosphate [182]

These facts tend to show that there is an important difference between natural hydroxylapatite and synthetic alpha tricalcium phosphate either pure or having adsorbed some calcium hydroxide Precipitates of tertiary phosphate called hydroxylapatite because of a Ca/P ratio of 2 14 do not actually have a complex molecule like natural hydroxylapatite, they represent only a purely physical association of tricalcium phosphate and calcium hydroxide

SUPPLEMENTARY REMARKS ON THE SECOND OPINION

This discussion calls for some supplementary remarks

Point a

First our theory is not incompatible with the fact that one can obtain beta tricalcium phosphate by calcinating at 900° C in presence of dicalcium phosphate tricalcium phosphate having adsorbed some calcium hydroxide that is the so called hydroxylapatite This fact has been demonstrated by Hirschman and collaborators [183] But it is true also if one uses natural hydroxylapatite instead of the physical association of tricalcium phosphate and calcium hydroxide

Point b

By mixing phosphoric acid and an excess of calcium hydroxide together with water at 100° C as has been done by Chaudron and Wallaeys [1?] one obtains a particular substance which does not have the same refractive index as alpha tricalcium phosphate (about 1 610 versus 1 590) This substance contains some water but also some calcium hydroxide which is bound to the molecule in a more intimate way than would be expected from a simple surface phenomenon This substance can also be obtained by boiling pure alpha tricalcium phosphate for a long time [184]

Point c

Let us also mention that precise measurement of the c/a parameters of natural hydroxylapatite carbonate apatite and alpha tricalcium phosphate reveals definite differences which permit making a further distinction between these substances [184]

These considerations are in favor of the actual existence of tricalcium phosphate as a crystalline chemical entity able to adsorb other mineral molecules

The Molecular Composition of the Bone Salt

It is very interesting to recall the historical development of studies and hypotheses concerning the molecular composition of the bone salt

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[183]Hirschman, A Sobel A E Kramer B and Fankuchen I An X ray Diffraction Study of High Phosphate Bones *J Biol Chem* 171 285 291 (1947)

[184]Brasseur H Dallemagne M J and Melon J Remarks on the Constitution of Hydroxylapatite and of Chlorapatite Synthesized by the Wet Method *Bull Soc R Sci Liège* 20 447 452 (1951)

HISTORICAL DEVELOPMENT

Berzelius one hundred years ago used the words "tricalcium phosphate" when he spoke about the bone salt. Discussions on the basic question actually started in 1862 when Hoppe[185] compared the composition of the mineral fraction of bone with that of the minerals related to apatite and found on quantitative analysis that the atomic ratios were similar in these two groups. The ratio $Ca/PO_4/CO_3$ is about 1.00/1.37/0.21 for bone and 1.00/1.42/0.15 for carbonate apatite.

Half a century later Gassmann[186] tried to bring more experimental evidence in favor of Hoppe's opinion. If this hypothesis was right, that is, if bone was actually made of carbonate apatite, the atomic ratio $Ca/PO_4/CO_3$ should remain constant in all circumstances. Gassmann declared this to be true after studying many samples of normal and rachitic bone. Marek, Wellmann, and Urbanyi[187] questioned this conclusion; according to them, tricalcium phosphate is chemically independent of bone carbonate. This has been confirmed many times since then; therefore the theory that the bone salt was carbonate apatite had to be dropped.

However, in 1926 the mineral substance of bone was examined for the first time by x-ray diffraction and the radiogram that was obtained was that of an apatite.[188] An interpretation was offered by Klement,[189] who stated that bone tissue contains hydroxylapatite associated with free carbonates. Klement used some very precise results collected by Gabriel[190] who had weighed the different mineral constituents of bone. From these results Klement tried to compute the elements of the structure of the molecule. He found the following ratio: $Ca_3(PO_4)_2/Ca(OH)_2/CaCO_3 = 3.00/0.99/0.4$, which is similar to that of hydroxylapatite: $Ca_3(PO_4)_2/Ca(OH)_2 = 3.00/1.00$.

In a long series of papers Klement brought arguments in favor of his

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[185] Hoppe, F. Studies on the Composition of Dental Enamel. *Virchow's Arch.* 24: 13 (1862).

[186] Gassmann, Th. Chemical Studies on Normal and Rachitic Bone. *Hoppe-Seyler's Z.* 70: 161 (1911).

[187] Marek, J., Wellmann, O., and Urbanyi, L. Chemical Syntheses of Bone Salts in Normal and Rachitic Animals. *Z. physiol. Chem.* 226: 3-17 (1934).

[188] De Jong, W. F. The Mineral Substance in the Bones. *Rec. Trav. Chim. Pays-Bas* 45: 445-448 (1926).

[189] Klement, R. The Composition of Bone Supporting Substance. *Z. physiol. Chem.* 184: 132-142 (1929).

[190] Gabriel, S. Chemical Studies on the Minerals of Bones and Teeth. *Z. physiol. Chem.* 18: 257 (1894).

hypothesis His explanation however, did not satisfy everybody and Silberstein[191 192] for instance, pointed out in 1935 that, if Klement's hypothesis were right, most of the CO_2 contained in bone would not be in a combined form, on account of the relative lack of cations Yet, the calculations done by Klement seemed conclusive

THE DISCOVERY OF AN ERROR

In 1943 I repeated the calculations of Klement[189] and noticed an error in them[173] In one operation, the decimal point had been misplaced, and the results arrived at by Klement were wrong In fact, the $Ca_3(PO_4)_2/Ca(OH)_2/CaCO_3$ ratio is not 3 00/0 99/0 4, but 3 00/0 71/0 7 which is definitely different from what should be found in the case of hydroxyl apatite 3 00/1 00

The whole question had to be reconsidered, and it is interesting to note that a misplaced decimal point can give rise to such arduous discussions as took place between Klement, Gassmann, Marek and others What Silberstein said was right In order to combine anions and cations in the form of molecules it is necessary to reject hydroxylapatite as the main bone constituent Only one solution seemed plausible tricalcium phosphate Of course, it was necessary to admit the actual existence of this substance, we have discussed this point before

Tricalcium Phosphate as the Principal Salt of Bone

If we admit that alpha tricalcium phosphate is a real chemical entity, we should now consider the arguments which make us think that this substance is the principal bone salt

METHODS FOR ISOLATING THE BONE SALT

First it is necessary to discuss the different physical and chemical methods which are used for isolating and studying the bone salt

Calcination

[illegible] only 500° C

[191] Silberstein L A Study of the Chemical Composition of Bone The Femur of the Horse *Compt rend Acad sc* Paris 200 421-423 (1935)

[192] Silberstein L Contribution on the Chemical Composition of Bone The Femur of the Horse *Bull Soc chim biol* 17 667-670 (1935)

Boiling and Tryptic Digestion

The method used by Bell and collaborators[193] consists in boiling powdered total bone for 24 hours and in submitting the remaining organic constituents to tryptic digestion However this method causes structural alterations (we have said that before about tricalcium phosphate) and an important loss of mineral elements [194]

Washing with Glycerine and Methyl Alcohol

The procedure employed by Gabriel[190] has the great advantage of using an anhydrous medium The mineral elements are not dissolved if they are carefully washed with glycerine and methyl alcohol Some structural modifications may occur but the radiogram is not altered Armstrong[195] was right in casting some doubt upon the value of the procedure but we do not know any better one There is no doubt that with Gabriel's method calcium citrate is changed into carbonate

One may wonder if in total bone calcium and phosphorus are really combined together Their combination might be the result of the destruction of the organic matter on which the atoms of calcium and phosphorus would be fixed individually in periodic alteration If this were true, fresh total bone probably would not give a radiogram corresponding to hexagonal or pseudo hexagonal crystallites Cartier[196] has shown that slices of sheep cartilage impregnated with calcium and pyrophosphate groups do not diffract X rays because the mineral elements are fixed on the preosseous organic matrix

It is also possible that in total bone tricalcium phosphate is bound to the organic chains and not to molecules of H_2O This could explain the periodic structural characteristics observed by Caglioti[197] but it cannot be considered yet as a well established fact

ARGUMENTS FOR ALPHA TRICALCIUM PHOSPHATE AS THE PRINCIPAL BONE SALT

Alpha tricalcium phosphate is the principal element of the bone salt isolated by Gabriel's method[190] The following arguments justify this opinion

Point 1

The results of quantitative analysis are compatible with the hypothesis that the bone salt is composed of tricalcium phosphate having adsorbed other mineral elements calcium and magnesium carbonates as well as sodium and potassium carbonate (or bicarbonates)

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193 Bell G H Chambers J W and Dawson I M The Mechanical and Structural Properties of Bone in Rats on a Rachitogenic Diet *J Physiol 106* 286 300 (1947)

194 Beaulieu M Brasseur H Dallemagne M J and Melon J Review of Methods of Mineralization of Bone *Arch internat physiol* 57 411 418 (1950)

195 Armstrong W D Composition and Crystal Structure of the Bone Salt *Trans Macy Conference on Metabolic Interrelations* 2 11-31 (1950)

196 Cartier P Personal communication (1951)

197 Caglioti V On the Structure of Bone *Atti I congr naz chim pura applicata Rome* Pt 1 320 330 (1936)

Point 2

The radiograms of bone and tricalcium phosphate are identical

Point 3

The refractive indices of the bone salt and alpha tricalcium phosphate have approximately the same value

Point 4

Calcination of the bone salt at 900° C results in the formation either of hydrxoylapatite or of carbonate apatite depending on whether CO can or cannot escape from the substance After calcination the refractive index has a higher value and it becomes impossible to separate the carbonate fraction from the phosphate fraction by any chemical method These facts can be confirmed by the study of the *c/a* parameters of the radiogram

Point 5

loss of weight it is easy to compute the amount of water eliminated as the total loss of weight is equal to the loss of H O plus the loss of CO_2 The amount of water eliminated from each sample is plotted against the temperature (Figure 31) The same procedure is u ed with samples of synthetic alpha tricalcium phosphate

phosphate we have observed that the relative amount of water contained in tricalcium phosphate is greater when the salt has adsorbed some calcium hydroxide or some carbonate

water fixat
calcium oxi
tricalcium
water is all
higher the water released by tricalcium phosphate combines with calcium oxide to form calcium hydroxide in this way the synthesis of hydroxylapatite is made possible Such results are obtained only if the bone salt very finely powdered is heated in an oven ventilated so as to permit the elimination of CO

This observation concerning the kinetics of the formation of true hydroxyl apatite is a fundamental argument in favor of the opinion that tricalcium phosphate is the main constituent of the bone salt

The Nature of the Bone Carbonates

Logan and Taylor[198] have observed that mineralized bone[199] treated with

[198] Logan M A., and Taylor H L. Solubility of Bone Salt III Partial Solution of Bone and Carbonate containing Calcium Phosphate Precipitates *J Biol Chem* **125** 391 397 (1938)

[199] Bone salts obtained by Gabriel s method[19]

dilute hydrochloric acid at 100° C releases more or less selectively part of its carbonate more than 50 per cent of the total amount of the carbonates is dissolved against only 10 per cent of the phosphates The solution contains a large amount of magnesium and sodium together with some calcium However it cannot be safely asserted that these elements were exclusively bound to CO_2 in mineralized bone as part of the phosphates also have been dissolved

We have found a method which provides a more selective separation of the carbonates from the phosphates With this method, the alkaline earth carbonates are changed into oxides these are soluble in water while the phosphates are not Bone samples are progressively heated up then immersed into water free from CO_2 The final solution now contains the elements which were bound to CO_3 groups in the original substance This accounts for most of the sodium and magnesium present in bone as well as for a small fraction of the calcium[200 201] (For additional discussion of these studies see Page 220)

Conference Discussion

Armstrong Thank you Professor Dallemagne We appreciate your very clear and complete presentation Are there questions or brief comments? As I said earlier I think the discussion would be most expeditiously carried out if the three main performers would have an opportunity to occupy the center of the stage without entering into prolonged discussion at this moment It would be in order however to put questions of fact or interpretation

Hamilton How are the bone salts obtained for these studies on dehydration? In Figure 31 how did you obtain the bone salts?

Dallemagne By Gabriel s method that is to say by heating total bone to 290° in glycerine with 6% potassium hydroxide for a half hour[199] and then washing with methyl alcohol

Armstrong Is this a procedure which hydrolyzes and solubilizes the proteins?

Dallemagne Yes

Meyer The glycerine is washed away but is there any nitrogen left?

Dallemagne No the nitrogen is gone

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[200]Dallemagne, M J and Melon J The Chemical State of the Magnesium and the Sodium of Bone Salts *Arch internat physiol* 58 188 200 (1950)

[201]Dallemagne M J Unpublished data

Hendricks The residual nitrogen content is usually of the order of a half per cent isn't it?

Hodge No

Dallemagne No we find no nitrogen

Hendricks Oh there is some surely

Armstrong I have carried out the procedure and there is no detectable nitrogen in the residue

Hendricks On teeth, Dr Armstrong or on bone?

Armstrong On teeth My experience has been with teeth

Hendricks It is very much easier I think to remove the nitrogen from dentin and enamel than it is from bone

Dallemagne No It is just the opposite It is easier to remove the nitrogen from bone than it is from teeth because teeth contain organic matter which cannot be destroyed by potassium hydroxide This organic component is probably a keratine

Armstrong Do you want to make any comments now or later with regard to the evidence obtained from the differential solubility of the components of the bone salt in acid?

Dallemagne I have not made new experiments in that respect However the solubility in acid is very different for fresh bone treated by the Gabriel method compared with calcinated bone salt

McConnell May I ask one question of detail please? Did I understand correctly that when you heat the tricalcium phosphate at some such temperature as 600° water recombined with the calcium oxide? That would seem strange because the decomposition temperature of calcium hydrate is below 600°

Dallemagne It is indeed a surprising fact that calcium oxide blocks water at such a high temperature

McConnell Well calcium oxide is a very good getter for water It will grasp any water in the immediate vicinity But the decomposition temperature of calcium hydrate, $Ca(OH)_2$ should govern the maximum temperature at which the water would be retained

Dallemagne It seems that the water can be blocked in the calcium hydroxide because of the combination of this compound with the tricalcium phosphate during the treatment in the oven

McConnell Then you are postulating essentially a hydroxylapatite structure

Dallemagne Yes At first, without any doubt, alpha tricalcium phosphate has an apatite structure The best argument about this results from x ray diffraction studies Moreover during the heating, the calcium hydroxide is progressively introduced into the lattice of alpha tricalcium phosphate and at this moment the compound becomes stable up to a high temperature

Neuman I would like to interject one comment which I think does not have any bearing on the conclusions reached Robert Kunin[202] pointed out that this water released around 400° to 600° corresponds to the dehydration of the hydronium ion, this suggestion leads to interesting speculations

[202]Kunin R Unpublished data

THE CRYSTAL CHEMISTRY OF FRANCOLITE AND ITS RELATIONSHIP TO CALCIFIED ANIMAL TISSUES

DUNCAN McCONNELL

From the Department of Mineralogy College of Engineering The Ohio State University Columbus Ohio

Armstrong Dr McConnell is next Most of you know that Dr McConnell is the originator and the chief proponent of the concept that carbon is a constituent of the apatite lattice I would now like to ask my friend and former colleague Dr McConnell to present the evidence for his interpretation of the constitution of bone mineral

McConnell Thank you Dr Armstrong for not saying the only proponent of this theory (Laughter) I hope my comments will be a little more comprehensible to you than some of your comments of yesterday particularly on collagen were to me—if I may be pardoned for a slightly facetious remark

The foremost question on the composition and crystal structure of calcified tissues is whether or not the carbon dioxide is contained within the crystal lattice of the apatite like substance Several important types of evidence will be presented in order to justify the conclusion that carbon dioxide as CO_3 groups occurs within the crystal lattice of the mineral francolite and therefore could occur in the lattice of the substances which make up dentin dental enamel and bone

Before we discuss the evidences that indicate CO_2 as a lattice constituent of francolite it seems advisable for us to examine some of the data which have led to the conclusion that CO is not a lattice constituent of calcified tissues

At the Second Conference on Metabolic Interrelations two years ago Dr W D Armstrong [203] presented an excellent summary of the formulas which have been applied to these calcified tissues Most investigators have assumed that dentin dental enamel and bone are qualitatively identical but quantitatively different This assumption will be retained in the present discussion and further justification for this assumption will be presented

The Problem of Isolating the Mineral Phase Without Modifying It

If we admit as an initial premise that the substances of dentin and bone

[203] Armstrong W D Composition and Crystal Structure of the Bone Salt *Trans Macy Conference on Metabolic Interrelations* 2 11 31 (1950)

are not single phases[204] but are mixtures of organic and inorganic compounds the problem of examining one phase in the absence of all other phases may become extremely difficult This is particularly true if the crystalline phase of interest happens to have a limited range of stability with reference to interaction with gases and liquids at various temperatures

Considering the comparatively minor variations which occur in the bodies of vertebrate animals with respect to the concentrations of dissolved inorganic salts, and with respect to pH, temperature, and so forth, one might surmise that calcification takes place within a limited range of conditions and that the calcified tissues have limited tolerance toward pronounced changes Therefore one would hardly expect to heat calcified tissues or bring them in contact with acids or caustics without significantly altering their physical and chemical properties Consider the simple compound $CaCO_3$ for example Two forms of this compound commonly occur naturally, as the minerals aragonite and calcite The former occurs as orthorhombic crystals and represents the major phase of the shells of most *mollusks* Calcite is far more common and is the essential component of limestones and marbles as well as the shells of fossil mollusks Paleontologists do not conclude that fossil mollusks employed different biochemical processes in shell formation, but merely that aragonite is a less stable form of $CaCO_3$ and is converted to calcite under the conditions of rock formation The analogy has limited value but it raises a significant question is it possible to separate the mineral phase of tooth or bone substance from the organic substances present without in any way altering the physical or chemical properties of the inorganic phase? Likewise one can ask has the unmodified mineral phase ever been examined in the absence of contaminating substances and are its physical and chemical properties accurately known?

Although obviously it would be simpler to confine the present discussion entirely to francolite (a mineral substance which occurs in small, clear, transparent, homogeneous crystals), this is not entirely possible because many of the data have been obtained on the more complex, heterogeneous substances of calcified tissues Two important sources of information are the data bearing on the solubility in acid and on the behavior on heating These evidences must be examined in light of the general properties of minerals

[204] A phase is an individual chemical substance with definite physical properties separable (in theory at least) by mechanical processes from other phases occurring in the same system It may be a crystalline solid a glass a molten liquid, an aqueous solution, or a gas

Information Obtained from Dissolution of Calcified Substances in Acid

Most silicate and phosphate minerals have very limited solubility in water and dissolution is differential that is the stoichiometry of the solid phase is not preserved in the liquid phase L C Armstrong[205] for example found that feldspar dissolves incongruently in water The velocity of removal of the alkalies or the alkaline earths is significantly greater than that of the aluminum and the silicon The known structure of feldspar indicates that the energy of bonding of sodium potassium and calcium in the feldspar is less than the energy of bonding for the aluminum and silicon and thus that the former would be expected to be liberated more rapidly Unfortunately it is no simple matter to predict atomic configurations from the mode of dissolution of the chemical constituents of a mineral and in the case of carbonate apatites it even appears to be uncertain from dissolution data whether or not the carbonate component is in the lattice At the same time it should be pointed out that the dissolution rate of CO_2 from francolite is quite different from the dissolution rate of CO_2 from a mixture of calcite and fluorapatite and that Silverman Fuyat and Weiser[206] have employed this empirical difference to estimate the calcite content of rock phosphates However since the bone salt cannot come into equilibrium with a mineral acid (such as HCl) we can assume that the solubility data will not yield valuable information on the structural environment of the CO_2

Information Obtained from Behavior on Heating of Calcified Substances

Several investigators including Dallemagne[207] and Sobel[208] and their respective co-workers have reported that the bone salt is converted to anhydrous tricalcium phosphate at some temperature above 600° C having lost its CO_2 content in the process The fact that a mixture of calcium carbonate and tricalcium phosphate behaves similarly is taken to indicate that the bone salt is a similar mixture This conclusion does not follow for reasons which can be illustrated by general mineralogical principles

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[205] Armstrong L C Decomposition and Alteration of Feldspars and Spodumene by Water *Am Mineral* 25 810 820 (1940)

[206] Silverman S R Fuyat R K and Weiser J D A Method for Distinguishing Free Calcite from Structurally Held Carbonate in Carbonate Apatite (Abstract) *Program of Winter 1951 Meeting Am Crystallogr Assn* Washington D C p 34 (1951) [Also see *Am Mineral* 37 211 222 (1952)]

[207] Dallemagne M J Brasseur H and Melon J The Constitution of the Mineral Substance of Bone and Synthesis of Apatites *Bull Soc Chim France Premier Trimestre* 16 D138 145 (1949) (Also see earlier references contained in this work)

[208] Sobel A E The Local Factor in Calcification, *Trans Macy Conference on Metabolic Interrelations* 2 113 143 (1950) (Also see earlier references contained in this work)

Let us consider the isomorphic series $Ca_3Al\ (SiO_4)_3$—$Ca_3Al\ (H_4O_4)_3$ the members of which form cubic crystals the temperature of significant decomposition for the former is about 1000° C for the latter apparently less than 350° C [4, 9] and for the intermediate isomorphic variants would be expected to be in the intermediate temperature range Among the apatites the fluorine containing compounds are the most stable and hydrated compounds would be expected to decompose at lower temperatures regardless of the presence or absence of CO_3 groups within the structure If as will appear later hydrogen can enter positions within the structure of apatite which are normally occupied by phosphorus the temperature of decomposition for a hydrated carbonate containing calcium phosphate with the apatite structure might be appreciably less than that for francolite

The Concept of Isomorphism and Carbonate Apatite

While we are considering the general mineralogical principles which are applicable to francolite and which likewise may apply to calcified tissues particularly dental enamel it is pertinent for us to discuss the concept of isomorphism Plagioclase feldspar one of the common rock forming minerals is not a simple compound in the usual sense of the chemist but is intermediate in composition between $NaAlSi_3O_8$ and $CaAl\ Si\ O_8$ Thus the calcium ranges from zero to one atom while the sodium ranges from one to zero and the silicon is replaced by aluminum to the same extent as the sodium is replaced by calcium Therefore in the plagioclase feldspars of an igneous rock no definite ratio between either the calcium and sodium or between the aluminum and silicon would be expected and any simple ratio that happened to occur would be fortuitous

For exactly the same reason one would not expect a constant or simple ratio for the calcium to phosphorus to carbon in a carbonate apatite if the carbon is capable of substituting for phosphorus or calcium or both Nevertheless this lack of constancy of the Ca P C ratio has been employed to support the conclusion that carbonate apatites do not exist even as minerals [210]

The Evidence Against a Submicroscopic or Colloidal Form of *Calcium Carbonate in Francolite*

While we are considering Ca P C ratios we should indicate quite clearly that if francolite were a mixture of submicroscopic or colloidal particles

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[209] McMurdie H F Personal communication (1951)

[210] Thewlis J Glock G E and Murray M M *Chemical and X Ray Analysis of Dental Mineral and Synthetic Apatites* *Tr Faraday Soc* 35 358 363 (1939)

of $CaCO_3$ and fluorapatite it would be a simple matter to deduct equal molecular amounts of CaO and CO_2 from the molecular ratio of francolite and thereby obtain the composition of fluorapatite. Since we do not obtain the composition of fluorapatite by such a deduction we must either assume that several mineral analysts of international repute have made unusually large analytical errors, or else conclude that francolite cannot be a mixture of fluorapatite and $CaCO_3$, whether the latter is in "submicroscopic" or in any other form.

It is well known that the x-ray diffraction lines of $CaCO_3$ have not been found in francolite or in normal calcified tissues, even though about 10 per cent of $CaCO_3$ by weight must be present if all of the CO_2 that has been determined by analysis is allocated to this compound. This significant negative evidence has been disregarded in several instances, and far more obscure evidence has been employed in reaching the conclusion that the $CaCO_3$ is present in a "colloidal" or "submicroscopic" form.

Comments on the Nitrogen Absorption Data of Calcified Substances

Recently Hendricks and Hill [11] have presented some interesting results based upon measurements of the nitrogen absorption and the solubility in acid of steamed bone, dental enamel and francolite. I have commented already on the validity of interpretations based upon the solubility in acid of calcified substances. Let us examine their nitrogen absorption data and ascertain whether or not alternate interpretations are available for these results. Undeniably the nitrogen absorption of the francolite was far in excess of what it should have been based on the normal external surface that would be produced by crushing the material to the sizes indicated by Hendricks and Hill. The steamed bone showed a remarkable constancy of area (by the B.E.T. method) for three sieve sizes: 4 to 8, 16 to 30, and 100 to 200. This is what would be expected for a highly porous material having pores large enough to be accessible to the nitrogen molecule. Midgley[12] recently has obtained similar data for opal and found 40 m²/g for 52 to 100 B.S. sieve particles. The broad diffuse x-ray diffraction bands produced by bone confirm the small size of the particles.

The surface areas calculated by Hendricks and Hill [11] for dental enamel and for francolite were quite different from that calculated for steamed bone. The areas changed by an appreciable factor for the finer particles and the maximum surface areas reported were 7.3 and 6.8 m²/g for fran-

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[11]Hendricks, S. B., and Hill, W. L., The Nature of Bone and Phosphate Rock. *Proc. Nat. Acad. Sci.* 36, 731-737 (1950).

[12]Midgley, H. G., Chalcedony and Flint. *Geol. Mag.* 88, 179-184 (1951).

colite and dental enamel respectively. Since the x-ray diffraction pattern of francolite will be discussed in greater detail later, it will suffice at present to recall that this substance does not produce broad, diffuse diffraction bands. Nevertheless, Hendricks and Hill have concluded that steamed bone, dental enamel and francolite are similar, that all three substances contain CO_3 adsorbed at the discontinuities within the crystals, and that the nitrogen absorption data support this conclusion. Although an explanation for the absorption data on francolite is not immediately apparent, a comparable absorption measurement for apatite would have been most interesting; it is unfortunate that Hendricks and Hill did not give this value for comparison.

The X-Ray Diffraction Data of Francolite

Quite in contrast with the experimental difficulties and the consequent possibility of error associated with the nitrogen absorption method is the simplicity and the high accuracy of the x-ray diffraction method. It is possible, for example, to compare the unit cell edges of francolite and fluorapatite, as well as the intensities of the numerous atomic planes within these crystals.

Precision measurements of the dimensions of the unit cells of francolite and fluorapatite were obtained at the National Bureau of Standards with the assistance of Mr. H. E. Swanson; these data are contained in Table XI. Inasmuch as the unit cell for francolite was less in the symmetrical direction *a* and only very slightly larger in the unique direction *c*, we conclude that the carbonate groups of Hendricks and Hill[211] cannot contribute to the average dimensions of the unit of structure. Nevertheless the unit cell of francolite is significantly different in size from the unit cell of fluorapatite.

TABLE XI

Comparison of the Unit Cell Dimensions (in Angstrom Units)

	a_o	c_o	c/a
Francolite (*Staffel, Germany*)	9.34_8	6.89_2	0.7373
Fluorapatite (Cerro de Mercado, Mexico)	9.39_5	6.88[illegible]	0.7325

(All data determined at the *National Bureau of Standards*)

The precision x ray data also revealed appreciable intensity differences for certain families of crystallographic planes Particularly the prismatic planes *(hk0)* show greater intensities for francolite than for fluorapatite The special prismatic reflections *(300)* are compared with respect to certain *(hkl)* reflections for these two substances in Figure 32 where complete re versals of relative intensities are shown

McConnell and Gruner[213] have pointed out that the optical properties of carbonate containing apatites are different from other apatites principally because of their higher birefringence Some years ago we were fortunate in obtaining from Magnet Cove Arkansas some samples in which a car bonate apatite occurs as a hydrothermal replacement product on the fluor apatite Figure 33 shows the overgrowth of the carbonate fluorapatite that is francolite on the common fluorapatite in a very nice fashion The large crystal which contains some inclusions and is a somewhat imperfect

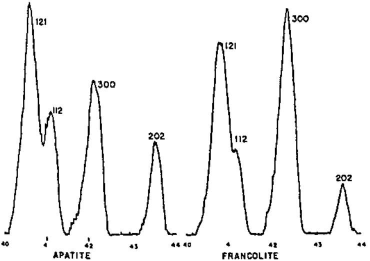


Fig 32 Comparisons of Intensities of Diffraction Spectra for Fluor apatite and Francolite for Diffraction Angles (Twice Theta) in the Region 40 to 44 Degrees

[Reproduced by permission from McConnell D The Problem of the Carbonate Apatites IV Structural Substitutions Involving CO_3 and OH Bull Soc Français e Mineral Cristal 75 in press (1952)]

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[213] McConnell D and Gruner J W The Problem of the Carbonate Apatites III Carbonate Apatite from Magnet Cove Arkansas *Am Mineral* 25 157 167 (1940)



Fig 33 Photomicrograph Showing Carbonate Apatite Replacing Fluorapatite

The carbonate apatite is the lighter substance the fluorapatite the darker Taken in polarized light with Nicol prisms in crossed position Magnification 24 X [Reproduced by permission from McConnell D and Gruner J W The Problem of the Carbonate Apatites III Carbonate Apatite from Magnet Cove Arkansas, *Am Mineralogist* 25 157 167 (1940)]

crystal has surrounding it in zonal arrangement another constituent the optical properties of which are different But what is even more clearly shown in Figure 33 is an overgrowth on the end in optical continuity if you please since the *c* axis is in the plane of the photograph we get the maximum birefringence and the retardation differences here are very significant indicating that the birefringence of the carbonate apatite is several times that of the ordinary fluorapatite

Now when we took these crystals and put them into a dilute hydrochloric acid solution it was readily apparent that the material with higher birefringence (the carbonate apatite) dissolved very rapidly liberating bubbles of CO_2 whereas the fluorapatite dissolved much more slowly and gave

no indication whatever of liberating CO_2. The optical properties then of francolite and fluorapatite are definitely different because in this instance there could not be a question of the retardation being due to differences in thickness, since the constituents of the thin section were all cut and ground in exactly the same way and the thickness was the same and the orientation was the same.

Also noteworthy is the fact that francolite is frequently biaxial and may show an optic angle as great as 40°.[214] The mean refractive index of francolite is slightly less than that of fluorapatite. However, there appears to be no straightforward correlation between the optical properties and the CO_2 content,[215] and this fact suggests that CO_3 groups enter the structure in more than one way or that some other constituent is also subject to isomorphic substitution within the structure. As will be explained later, both possibilities appear to occur.

For the present we shall be content to conclude that: 1) there are differences in the dimensions of the unit cells; 2) there are differences in the relative intensities of certain families of atomic planes; 3) there are differences in the densities; and 4) there are differences in the optical properties of francolite and fluorapatite.[16] Therefore, there are differences in the crystal structures of these substances. By this it is understood that the average unit cell of francolite contains different component atoms, and these atoms of necessity are arranged in slightly different ways. These conclusions have no bearing on any porosity which the crystals may have regardless of the method of measuring this porosity.

Before seeking an hypothesis capable of explaining some of these differences in the properties of francolite and fluorapatite, it might be well to mention one other observation obtained by X-ray diffraction methods. Although some small measure of defect is indicated in the francolite pattern resulting in partial loss of alpha one resolution, the widths of the diffraction lines are not noticeably increased. This observation completely fails to confirm the suggestion of Hendricks and Hill[21] that discontinuities of the order 0.1 micron exist in the francolite crystals. Likewise it should be mentioned that discontinuities with significantly larger spacings presumably would produce scattering of wave lengths within the visible range, but

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[214] Deans, T. Francolite from the Sedimentary Ironstones of the Coal Measures. *Mineral. Mag.* 25, 135-139 (1938).

[215] Hutton, C. O. and Seelye, F. T. Francolite, a Carbonate Apatite from Milburn, Otago. *Trans. & Proc. Roy. Soc. New Zealand* 72, 191-198 (1942).

[16] Gruner, J. W., and McConnell, D. The Problem of the Carbonate Apatites. The Structure of Francolite. *Z. Krist.* 97A, 208-215 (1937).

neither broadening of the x ray diffraction maxima nor visible scattering are observed for the small clear, transparent francolite crystals

The Chemical Analysis of Francolite

In an earlier attempt to explain the properties of francolite, Gruner and McConnell[216] developed a structural hypothesis which can now be regarded as incomplete It was based upon merely one high quality chemical analysis of francolite Other recent concepts concerning isomorphism of minerals were unknown at the time, so that the hypothesis of these investigators for francolite as well as the conclusions arising from their collaboration with Armstrong[217] on dental enamel and dentin now require modification

Five excellent chemical analyses of francolite from widely different geographical sources are now on record If a few simple crystal chemical assumptions are applied to such analyses, it becomes feasible to calculate the number of atoms of each kind which will occur in the average unit cell since these numbers need not be integers The detailed methods for making

TABLE XII

Summary Computations from Composite Analysis of Francolite

Electrical Equivalence			Spatial Equivalence		
Ca Mg Sr	2 × 9.89	19.78	Ca Mg Sr	9.89	9.89
Na	1 × 0.01	0.01	Na	0.01	0.01
H_2O	0 × 0.10	—	H_2O	0.10	0.10
P V	5 × 5.36	26.80			10.00
C	4 × 0.68	2.72	P, V	5.36	5.36
H	1 × 0.52	0.52	C	¾ × 0.68	0.51
		+49.83	H	¼ × 0.52	0.13
					6.00
O	2 × 23.88	47.76	O	23.88	23.88
F	1 × 2.12	2.12	F	2.12	2.12
		—49.88			26.00

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[217]Gruner J W McConnell D Armstrong W D The Relationship Between Crystal Structure and Chemical Composition of Enamel and Dentin *J Biol Chem* 121 771 781 (1937)

these calculations are given elsewhere,[217] only the summary results need be considered at this time (Table XII)

An Hypothesis for the Structure of Francolite

Hendricks and Hill[218] previously recognized the possibility that four carbon atoms might substitute for three phosphorus atoms in the crystal lattice

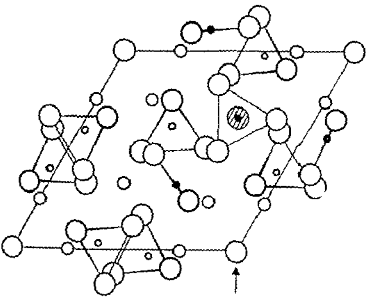


Fig 34. Projection of the Structure of Fluorapatite on the Basal Plane (001) Showing the Symmetrical Introduction of CO_4 Groups in Substitution for PO_4 Groups

The unit cell is outlined. *The large open circles* represent oxygen atoms except those on the unit-cell boundary lines which represent fluorine *the intermediate circles* represent calcium atoms *the small open circles* phosphorus *the small solid circles* carbon and *the large shaded circles* water molecules *The arrow* indicates the direction for viewing Figure 35 [Reproduced by permission from McConnell D The Crystal Chemistry of Carbonate Apatites and Their Relationship to the Composition of Calcified Tissues *J Dent Res* 31 53 63 (February 1952)]

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[217] McConnell D The Problem of the Carbonate Apatites IV Structural Substitutions Involving CO_3 and OH *Bull Soc Français Minéral Cristal* 75 in press (1952)

[218] Hendricks S B, and Hill, W L The Inorganic Constitution of Bone *Science* 96 255 257 (1942)

of apatite The present hypothesis retains this suggestion but places these carbon atoms in different coordinates and thereby obtains different effects with respect to the optical properties and the intensities of certain families of diffraction maxima A schematic representation of the arrangement of the carbon atoms in the unit cell of francolite is shown in Figure 34 and a more detailed representation of a portion of the atoms of the unit cell in Figure 35

THE LOCATION OF THE CARBON ATOMS

*In the proposed structure one fourth of all carbon atoms would be sur*rounded by oxygens in such a manner as to enhance the birefringence and the other three CO_3 groups would tend to alter the intensities of x ray diffraction maxima of the prismatic sort Qualitatively, these effects are consistent with the observed data Quantitative proof of the structure must

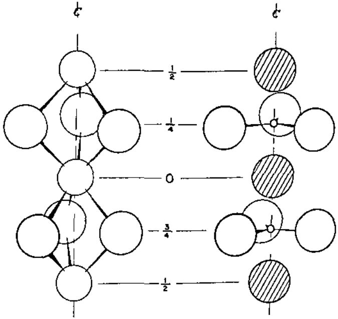


Fig 35 Clinographic Projection of the Atomic Arrangements *in the* Vicinity of the Three Fold Axis for Fluorapatite and Francolite

This figure is a view from the direction of the arrow in Fig 34 *Left* Fluorapatite with calcium atoms in six fold coordination *Right* Francolite indicating formation of a CO_3 group parallel with the basal plane (001) and replacement of the calcium atoms by water molecules The fractions represent the relative dimensions of the unit cell in the direction of the unique axis *c* [Reproduced by permission from McConnell D The Crystal Chemistry of Carbonate Apatites and Their Relationship to the Composition of Calcified Tissues *J Dent Res* 31 53-63 (February 1952)]

await far more accurate data on an analyzed sample of fluorapatite.

Data and conclusions regarding very slight alteration of the coordinates of the calcium atoms as presented by Bale[220] are completely meaningless when applied to an apatite crystal of unknown chemical composition. Beevers and McIntyre[221] decided that it was not the calcium coordinates that needed further refinement but the phosphorus coordinates. However, there is no indication that these investigators worked with analyzed material and therefore, although their conclusions are different from Bale's, no reliance can be placed on their results. Incidentally, their paper fails to give their newly determined coordinates for phosphorus.

THE LOCATION OF THE HYDROGEN ATOMS

The proposed hypothesis for the structure of francolite deals with the question of the location of carbon, to be sure, but it also takes into account the observation that all of the francolite samples that have been specifically tested contain water (or hydroxyl ions) which is liberated above 300°C. Although the chemical data examined by Gruner and McConnell[16] revealed this fact, the true significance was not appreciated by these authors at that time because the possible substitution of hydrogen atoms for such elements as phosphorus was not recognized.

One isomorphic series in which silicon is completely replaced by four hydrogen atoms has already been mentioned. Pabst has shown that $Ca_3Al_2(SiO_4)_2(OH)_4$ is isostructural with $Ca_3Al_2(SiO_4)_3$.[222] That phosphorus can substitute for silicon in this structure has been demonstrated by McConnell[223] for the composition $(Na, Al, Ca, Fe)_3Mn_2(PO_4)_2(OH)$. Additional examples of SiO_4 or PO_4 being replaced by H_4O_4 or H_xO_4 are found for viseite[224] and montmorillonite.[225,226] It is concluded, therefore, that although francolite has all of the fluorine positions filled with fluorine

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[220]Bale, W. F.: A Comparative Roentgen Ray Diffraction Study of Several Natural Apatites and the Apatite-Like Constituent of Bone and Tooth Substance. *Am. Roentgenol.* 43: 735-747 (1940).

[221]Beevers, C. A. and McIntyre, D. B.: The Atomic Structure of Fluor-Apatite and Its Relationship to That of Tooth and Bone Material. *Mineral. Mag.* 27: 254-257 (1946).

[222]Pabst, A.: The Crystal Structure of Plazolite. *Am. Mineral.* 22: 861-868 (1937).

[223]McConnell, D.: Griphite, a Hydrophosphate Garnetoid. *Am. Mineral.* 27: 452-461 (1942).

[224]McConnell, D.: Viseite, a Zeolite with the Analcime Structure and Containing Linked SiO_4, PO_4 and H_4O_4 Groups. *Am. Mineral.* 37: 609-617 (1952).

[225]McConnell, D.: The Crystal Chemistry of Montmorillonite. *Am. Mineral.* 35: 166-172 (1950).

[226]McConnell, D.: The Crystal Chemistry of Montmorillonite. II. Calculation of the Structural Formula. *Clay Minerals Bull. (London)* 1: 179-188 (1951).

hydroxyl ions or water molecules or both occur within the structure of francolite Furthermore, it is assumed that the crystal chemical calculations are capable of indicating the distribution of the necessary hydrogen ions in view of the principles of isomorphic substitution

Summary of the Thesis on the Structure of Francolite

These assumptions permit an indication of the remaining portions of the structural hypothesis together with some of the principal consequences and applications thereof The entire thesis can be summarized as follows

Point 1

The lattice of closely packed oxygens in francolite is essentially similar to that of fluorapatite

Point 2

The fluorine positions of fluorapatite are completely occupied by fluorine in francolite

Point 3

Carbon atoms substitute for phosphorus atoms but for each 12 oxygens thus involved four carbons replace three phosphorus atoms although their coordinate positions are different

Point 4

For each four CO_3 groups thus formed approximately three lie in a plane perpendicular to *(001)* whereas one is parallel with *(001)*

Point 5

The CO_3 groups lying parallel with *(001)* cause vacancies in certain calcium positions on the simple three fold symmetry axes

Point 6

Where vacancies in these calcium positions occur the space is filled by water molecules

Point 7

Some of the phosphorus containing tetrahedra of fluorapatite contain hydrogens in francolite

Point 8

The water liberated above 300° C from francolite represents recombination of these hydroxyls or liberations of water molecules substituting for calcium or both

Point 9

The CO_3 groups lying parallel with *(001)* cause the high birefrigence of francolite

Point 10

The CO_3 groups perpendicular to *(001)* contribute the observed intensity differences for the *(hk0)* planes of francolite

Point 11

The lesser density of franc[illegible] is not related to porosity but is produced by substitution of constituent[illegible] c weight

Point 12

The contraction in the *a* direction of the unit cell is the result of the increased polarization of the carbonate groups

Point 13

The very slight increase in the *c* dimension is the result of substitution of water molecules for calcium which occurs to a very small extent

Point 14

There is no constancy in the ratio of Ca P C to be expected nor is any found on analysis

Point 15

The heating of an unstable isomorphic variant containing water molecules and hydroxyl ions of the sort postulated for francolite could easily produce a transformation to beta $Ca_3(PO_4)_2$ if sufficient hydrogen were present in these forms

Point 16

If the quantities of water and hydroxyls were insignificant the transformation might not occur or might not be detectable

Point 17

Point 18

Since the substances of teeth and bones contain hydroxyl ions which correspond to the fluorine ions of fluorapatite in addition to the other hydroxyl ions and water molecules mentioned they will liberate larger quantities of water but they do not correspond to any definite hydrate such as the formulas proposed for alpha tricalcium phosphate hydrate

Point 19

The so-called 'alpha tricalcium phosphate hydrate may represent an isomorphic variant which is otherwise similar, but does not contain CO_3 groups Under these circumstances the Ca P H O ratios would be expected to differ slightly if the conditions of preparation were altered

Point 20

The decomposition temperature or transformation temperature of the so-called alpha tricalcium phosphate hydrate would be expected to differ according to differences in the Ca P H O ratios

Point 21

The conclusion that CO_3 groups are adsorbed on submicroscopic discontinuities within francolite crystals is inconsistent with the evidence obtained by x ray diffraction

Point 22

The differential dissolution data lead to ambiguous conclusions concerning the location of the carbonate content

Point 23

The thermal transformation data are similarly unreliable in determining the structural environment of atomic groups within a complex isomorphic variant particularly so, if the isomorphic variant is a complex hydrate

Point 24

The optical properties the density, and the chemical composition for the mineral phase of dentin and bone have not been determined for samples where an unaltered and uncontaminated condition has been demonstrated

Point 25

Precise data on chemically analyzed samples of fluorapatite are required before the quantitative differences between the structures of fluorapatite and of francolite can be resolved adequately by x ray diffraction methods

The preceding 25 statements are phrased without qualification for the sake of brevity Together they comprise the hypothesis for the structure of francolite and its relationship to the substance of calcified animal tissues Out of context any individual statement is probably misleading, it is certainly incomplete

COMMENTS ON THE CRYSTAL CHEMISTRY OF BONE

STERLING B HENDRICKS

From the Bureau of Plant Industry, Soils and Agricultural Engineering U S Department of Agriculture Beltsville Maryland

Armstrong I think is would be wise, before we amateurs open our discussion for us to hear from the other expert Dr Hendricks will you speak now, please?

Hendricks I don t know whether your definition of an expert is a man who really knows something about something or is just another man a long way away from Washington

Well as you know, I don t subscribe to what Dr Dallemagne and Dr McConnell have just told you That is one statement Somehow or other I therefore, either have to pick holes, little holes here and there or else have to give another broader point of view

Last week, I remarked that I didn't want to come to the Conference particularly because I had presented last year all that I knew about this subject Then I said to myself "What will Dr Dallemagne and Dr McConnell say? And I did write out quite accurately indeed and precisely what each of them has said That shows no particular astuteness on my part but it shows that if you go through these arguments in a particular sort of way, according to the classical concepts historically you will come out with the answers that you have been given In other words I essentially disagree with only one of the points that have been presented I spoke to Dr McConnell during the intermission about this one namely that the diffraction pattern of francolite is rather markedly different from that of bone salt

The Validity of Comparing the Data from Mineral Substances with Those from the Bone Salt

You have heard about the material, francolite about the mineralogical species of carbonate apatite, and about other substances of this sort and I think you might justifiably raise in your minds the question "What is the relationship, if any, of these materials to bone?" I believe that when you examine this question very thoroughly, you will find that these materials form entirely justifiably parallel cases for discussion The only reason that these substances are introduced into our deliberation at this time by Dr McConnell and by others is simply the fact that the geological processes for

their formation have been very much slower than the biological processes for the formation of the bone salt and as a consequence that you can obtain these materials in larger better defined crystals Because of such crystals you can carry out a more precise examination of their crystal properties and possibly can then determine the structures of these particular substances Similar considerations apply to tricalcium phosphate hydrate about which Dr Dallemagne spoke This is a material that has to be prepared synthetically again you might question the relationship if any of it to the bone salt Tricalcium phosphate hydrate is the type of material that you obtain from synthetic systems of CaO/P_2O_5 and water I believe it is entirely justifiable to introduce this synthetic material because the phenomenon involved in it also enters into bone

Now I have to present some very difficult matters, and go quite deep into some crystallographic subjects I will try to do this with the least possible torture I am going to attempt to explain to you the nature of the structure of a crystal because that is involved here I am going to try to show you the nature of crystal optics because that too is involved here And then finally I want to show you something very odd about another compound which is not directly bone but which will illustrate these phenomena too

An X Ray Diffraction Pattern of Francolite

Dr McConnell discussed an x ray diffraction pattern of francolite I want to challenge the validity of this particular pattern In my opinion the principal reason that the sample of francolite gave this particular pattern was due to crystal orientation I would like to read another statement from Dr McConnell This is from Gruner and McConnell[227] about the structure of francolite They state the following The photographs of almost pure fluorapatite and francolite agree so well with one another that only on close inspection are certain differences revealed That I think is a true statement To have a photograph of a specimen that is oriented is not unusual because of the difficulties for instance in mounting the specimens for this type of diffraction pattern To let the matter stand however would challenge almost all of the literature which states that the diffraction patterns *of all of these materials are essentially similar* Such statements have been made among others by Bale Hodge Hendricks and Hill Thewlis Tromel Dallemagne and Brasseur McConnell Gruner and Armstrong You see I quote the people at this Conference Such statements were made also by Tovborg Jensen and Moller[228] among others

[227]Gruner J W and McConnell D The Structure of Francolite *Z Krist* 97A 208 215 (1937)

[228]Tovborg Jensen A and Moller E Investigations in the Properties of Tooth Enamel by Means of X Rays *Acta odon scand* 6 7 18 (1944)

In answer to my objection, Dr McConnell says nevertheless that he believes that the diffraction pattern is correct as he stated it so we are left with a clash of facts Now, Tovborg Jensen and Eva Moller[28] say "The lattice constants of hydroxylapatite were found to be $a = 9.431$ and $c = 6.857$, in excellent agreement with Thewlis but deviating greatly from Burri The same value was found for dental enamel, that is the same value of the glancing angle was found for enamel and for hydroxylapatite but as enamel is a colloid and the lines of a large, scattering angle cannot be measured the lattice constants of enamel are determined with less accuracy" *It is the precise point that led Hodge and Bale*[29,30] *to say* that bone is hydroxylapatite, because they never saw any difference in diffraction pattern between the bone salt and synthetic hydroxylapatite This is the underlying reason We examined this point a little bit here last year and we want to examine it again

The Structural Features of Crystals

Now, what is a crystal? A crystal structure is the pattern, such as Dr McConnell has given in Figure 34, illustrating the projection of the atomic arrangement upon a plane The atomic arrangement is defined in terms of a small parallelepipedon It is like a cigarette pack If the volume of the pack is reduced by a hundred million fold, you will have a unit that is adequate to describe the structure of hydroxylapatite Within this small unit, which is the unit of structure, the atoms are repeated according to the symmetry elements, and those symmetry elements were figured out in classical crystallography long before x-ray diffraction was discovered They were figured out accurately in 1895, as a matter of fact before any use could be made of them, from the observation that crystals have certain external forms It followed that crystals could be described by group theory There are 230 different methods of describing them

Hydroxylapatite belongs to a particular crystal system It has a particular external symmetry of its faces Therefore, the one problem is to find the atomic arrangement within this little unit, and that is what we call determination of the crystal structure After you have determined the structure of this unit you can build up the entire crystal by translational repetition of this unit

In other words, there are two structures here There is that of the lattice which is built up by translational repetition of the smallest unit and that

[29] Hodge H C, and Bale W F The Molecular Constitution of the Calcium Phosphates *J Biol Chem* 123 lvii (1938)

[30] Hodge H C Some Observations on the Dynamics of Calcification, *Trans May Conference on Metabolic Interrelations* 2 73 112 (1950)

of the smallest unit which is the basic feature that determines the structure of the crystal X ray diffraction is dependent on both of these structures It is determined by the structure within the unit and it is also determined by the repetition It is the repetition that gives the lattice constants such as were shown for francolite The lattice constant *a* might be one dimension the lattice constant *c* might be another dimension Actually apatite has a hexagonal unit while the cigarette pack has an orthorhombic unit There is one additional point which is a little more astute and which goes a little deeper into the nature of mathematical crystallography that is within a unit a hexagonal one for instance crystals are built up according to hexagonal symmetry as a subgroup of operations that are monoclinic They are of lower symmetry

The Nature of Crystal Optics

Now what about the crystal optics? Well the crystal optics also are based upon the symmetry of crystals In the discussion that has been given it was correct to refer to the crystal optics that have been mentioned here The crystal optics are measured in a very fundamentally different way The diffraction pattern of the structure of a crystal is taken with x rays and the reason x rays are useful for these measurements is that x rays have wave length dimensions of the order of the distance between the atoms and the lattice The reason you get a diffraction pattern is that there is a regular repetition of the units and it is the regular repetition that gives the diffraction pattern The crystal optics as I say also owe their origin to that crystal regularity However they probe in a much different type of phenomenon They probe with a wave length of the order of 1000 to 10 000 atomic dimensions Wave lengths of the order of 1000 of these units of structure are required to describe this material in microscopic dimensions

You can see immediately that crystal optics will average the phenomena *that are taking place within the crystal The x ray* diffraction will not so greatly average the phenomena It will measure more precisely what is going on within the unit of structure The optics will more nearly measure what is going on in the so called real crystal that is across the dimensions of the crystal as a whole

The Mol Refractivity of Hydroxylapatite

Now I want specifically to go rather deep into crystal optics for the moment and in so doing I will indeed show that the particular structure that Dr Dallemagne mentioned for tricalcium phosphate is really impos

sible It is not debatable, it is quite impossible [31] The point is this that the optical properties of a crystal depend upon the polarizability of the atom I can illustrate this if you wish A wave hits the atom displaces an electron and sets it into forced vibration the degree of which is a property of the atom The properties of the atoms are such that those atoms which are negative and those which are more loosely made contribute most to the index of refraction Specifically you can take Landolt Bornstein or any set of tables of physical constants and you will find there the atomic refractivities of materials or you can find these constants in Bragg's[32] book on the structure of minerals if you wish Some atomic refractivities which have the dimensions of a volume are calcium—2 cm³ oxygen—3 50 cm³, and hydroxyl—same as oxygen Fluorine has essentially the same polarizability as oxygen There is a little uncertainty in these values but they are relatively precise The highly charged ions such as phosphorus and carbon whatever the charge on the carbon might be are low and you might take them as 0 40 cm³ If you calculate the mol refractivity of hydroxylapatite, which has been correctly written as $Ca_{10}(PO_4)_6 \bullet (OH)_2$ by adding the atomic refractivities you get 113 4

There is a different method however and quite a classical method of finding the value for the mol refractivity This is to derive the values from other compounds The calcium figure might have been derived from calcium chloride or calcium oxide, the oxygen figure from magnesium oxide—totally unrelated substances These values are atomic constants and the crystal in which you place them makes no essential difference in their value

The atomic constants are obtained from what is called the Lorenz Lorentz formula' and I believe all of you who are teaching probably present it to your students The 'Lorenz Lorentz formula says this that the mol refractivity—this is a pedagogical session you see—is equal to the molecular volume times N squared minus one over N squared plus two where N is the average refractive index In other words

$$\text{mol refractivity} = \frac{\text{molecular weight}}{\text{density}} \bullet \frac{N^2-1}{N^2+2}$$

This value which proves to be 116 cm³, can be used to calculate the mol refractivity of hydroxylapatite The difference between this value and the value of 113 4 obtained from the atomic refractivities is very small and is

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[31] This approach has been developed by A S Posner and S R Stephenson of the National Bureau of Standards

[32] Bragg W L Atomic Structure of Minerals Cornell Univ Press Ithaca N Y, p 120 (1937)

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There is a different method, however, and quite a classical method of finding the value for the mol refractivity. This is to derive the values from other compounds. The calcium figure might have been derived from calcium chloride or calcium oxide, the oxygen figure from magnesium oxide—totally unrelated substances. These values are atomic constants and the crystal in which you place them makes no essential difference in their value.

The atomic constants are obtained from what is called the Lorenz-Lorentz formula, and I believe all of you who are teaching probably present it to your students. The Lorenz-Lorentz formula says this: that the mol refractivity—this is a pedagogical session, you see—is equal to the molecular volume times N squared minus one over N squared plus two, where N is the average refractive index. In other words

$$\text{mol refractivity} = \frac{\text{molecular weight}}{\text{density}} \cdot \frac{N^2-1}{N^2+2}$$

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[31] This approach has been developed by A. S. Posner and S. R. Stephenson of the National Bureau of Standards.

[32] Bragg, W. L., Atomic Structure of Minerals, Cornell Univ. Press, Ithaca, N. Y., p. 120 (1937).

accounted for if oxygen has the value 3 60 instead of 3 50—which is permissible in this sort of calculation But don't get the idea that this is an inexact quantity It is an exceedingly exact quantity—the mol refractivity of hydroxylapatite is 116 cm ^{3}

The Mol Refractivity of 'Tricalcium Phosphate Hydrate'

Now, where do we go from here? Let us consider 'tricalcium phosphate hydrate' Dr Dallemagne mentioned the indices of refraction of these materials You will note that oxygen largely determines the index of refraction It is for this reason among others that the suggestion of Dr McConnell that water is added in the position of calcium in the so called 'carbonate apatite lattice is impossible It is for this reason that the suggestion which was advanced by Dr Dallemagne for the structure of the 'tricalcium phosphate hydrate' is untenable More specifically, one of the indices of refraction of hydroxylapatite is 1 645, and the other, 1 650 The index of refraction of tricalcium phosphate can be as low as 1 590

The diffraction pattern of 'tricalcium phosphate hydrate' is closely the same as that of hydroxylapatite, which means that the molecular volume is the same for both substances Now, if the mol volume is altered within the small limits proposed by Dr McConnell the resulting change is only of the order of a small fraction of a per cent A change of not more than 0 5 cm ^{3} in the mol refractivity would be accounted for by any of the suggestions that have been made for an alteration in mol volume, and the change might be in either direction You will note immediately that the value for the mol refractivity of the 'tricalcium phosphate hydrate' would have to decrease since the index of refraction of the compound is 1 590 Now Dr Dallemagne did not use the refractive index except *to say on* the basis of it that the material differs from hydroxylapatite Dr McConnell also used the refractive index only as evidence upon which to say *that this material is different,* without *analyzing* what the observed value actually means It actually means that the value for the mol refractivity must be decreased considerably, and if you go through and calculate what does happen, the mol refractivity value is decreased from 116 to 107 5 The difference is 8 5 cm ^{3} This shows quite clearly that oxygen must be left out of the lattice in the structure of 'tricalcium phosphate hydrate' The structure proposed by Dr Dallemagne did not leave oxygen out of the lattice, it rather substitutes hydroxyl groups or water for calcium

You can look at this in another way A volume changed since the refractivities have the dimension of a volume Upon calculation you find that the molecular volume of 'tricalcium phosphate hydrate,' instead of being 318 5 as for hydroxylapatite, apparently is *372* The density of the

"tricalcium phosphate hydrate" correspondingly dropped to 2.88, which is approximately the observed value. If the formula of the material within the unit of structure is to be written, it is $Ca_{8.47}(PO_4)_{6}(H_2O)_{4}$. In other words, to explain the observed refractive index, phosphate must have been left out of the unit structure as well as calcium. There is no alternative.

The Mol Refractivity of Carbonate Apatite

You can go through all of these arguments with carbonate apatite, and they are not quite so striking. The index of refraction of "tricalcium phosphate hydrate" dropped from the order of 1.640 to 1.650 for hydroxyl apatite down to the order of about 1.565 to 1.590—a tremendous drop. The refractive indices of carbonate apatites did not drop so markedly. However, the refractive index of carbonate apatite does decrease, as Dr. McConnell will verify. The refractive index of carbonate apatite decreased from the order of 1.640 to 1.650 for hydroxylapatite down to the order of 1.630 or 1.620. If anything happened, the atomic volume decreased somewhat.

This is the way to state the conflict. There is a void in the system. One group of people choose to say that the void is within the unit of structure. The others, namely myself in this case, choose to say that it is outside the unit of structure. This is the whole conflict. In other words, you can quite precisely show that there are voids in the system because the refractive indices dropped; one group says that the void occurs within the unit, and the other says that it occurs between the units in some way.

Now the particular elaborate composition that Dr. McConnell shows for fluorapatite in Figure 34 is based upon the assumption that the oxygen atoms of all the phosphate groups were there, and, in addition, hydroxyl groups or water substituted for calcium. Such a substitution which involves the addition of oxygen would raise the refractive index. Actually, what is observed? The density drops and the refractive index also drops. It is in this way that voids appear in the system. The only question is whether these voids appear in the unit of structure or whether they appear outside the unit of structure. Of course, there are many points, minor points, on which one could quibble.

The Evidence Obtained from Anisotropism

I want to introduce a different property of crystal optics. Many crystals are anisotropic, that is, the index of refraction depends upon the direction in the crystal. True enough, francolite, which is a perfectly justifiable material to use, is apparently biaxial. What does the nature of the biaxiality mean? Let me draw your attention to the fact that sodium chloride, which

is supposed to be a material that is isotropic often has a birefringence The mineralogist and the petrologist have often solved this by a beautiful method *They have called* it anomalous By taxonomy you see they have conquered the phenomenon (Laughter)

The other point about it is that many hexagonal crystals have a tendency to be anomalous one of these for instance is the mineral beryl and another is the mineral aragonite I went back and dug up the old data that we had taken in 1933 on single crystals of dahllite which is one of these *carbonate apatites A large crystal of dahllite gives a uniaxial interference* figure but fragments from a broken crystal are often biaxial Moreover dahllite has the important property of being very variable in its biaxial character as is known from the observations of Lacroix[233] He worked particularly with a material known as quercyite from a locality in France It was so variable as to suggest more than one form The alpha form was optically positive and the beta form optically negative or the other way around I don t recall which In other words it was extremely variable This variability itself is a striking property since most crystals with exact structures do not have such exceedingly variable optics

Actually the birefringence which is the difference in refractive indices will show you another thing In the carbonate group the carbon atom and the three oxygen atoms are all in the same plane, because of this when the electric vector is perpendicular to the plane the optic *properties are* markedly different from when the vector is in the plane of the group It is for this reason that calcite has such an exceedingly high birefringence *The carbonate group imparts a very high* birefringence to any crystal in which it enters unless it has a number of different orientations There are cubic carbonates for example But if the carbonate group has a single orientation or if it were as Dr McConnell says in the plane of the group perpendicular to the hexagonal axis the birefringence would be of the order of 0 04 instead of about 0 01 as observed The lower birefringence that is observed indicates that the carbonate group has a number of different configurations *These are simple deductions from the crystal optics*

The Parallel Case of Conchite

There is another point If I were to stop now you would all say Well it is a matter of paying our money and making a choice Which is it? You don t know You are not all experts in this field You can hear one man and consider his tale logical *and you hear another* and equally believe him The position possibly would be better if one found an exact

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[233] Lacroix, A *Minéralogie de la France et de ses Colonies* Paris 4 555 (1910)

analog with the characteristics that have been discussed Francolite and tricalcium phosphate have been used to some extent as analogs Accordingly I looked for an analog so convincing that the mineralogists and crystallographers here would say Yes darn it that s true! (Laughter) Fortunately one turned up very quickly It turned out to be the calcium carbonate of mollusk shells which in a sense has a setting equivalent to bone

Mineralogists have been working on the material of mollusk shells ever since 1895 They called the material conchite What are the crystal properties of conchite? Conchite is an aragonite which is one of the two common crystal modifications of calcium carbonate In mollusk shells it is very pure Calcite the other common crystal modification of calcium carbonate also appears in some mollusk shells and in other animals that form shells In this substance however the calcium has the property of taking quite a lot of magnesium into the lattice

What is this property of conchite that is exactly parallel to that of the mineral francolite? The property is that its refractive indices differ considerably from those of common aragonite just as the refractive indices of francolite differ from those of fluorapatite In addition conchite gives so precisely the diffraction pattern of common aragonite that it is now called a discredited mineral species For that reason this mineral conchite or the mineral name, conchite dropped out entirely But the crystal optics are exceedingly amusing Common aragonite has the refractive index alpha equal to 1 5331 The value is exceedingly precise and I want to write all the known figures The beta index is 1 6816 and the gamma index is 1 6859 The average refractive index [and don t add up these three and divide them by three because that isn t the way to get the average (laughter)] is 1 635 Mollusk aragonite or conchite has alpha 1 523 beta 1 659 and gamma 1 662 with an average index of 1 616 As the material has the same x ray diffraction it has the same molecular volume The refractive index however is lower which means that the mol refractivity must be lower

The crystals give some evidence as was the case in dahllite of unusual properties This conchite form of aragonite instead of showing the usual parallel extinction of these sorts of materials shows a continual variation for the extinction position along an aggregate The actual case then is that the conchite form of aragonite as it appears in mollusk shells is an exact parallel with the material in bone The case however is much simpler since the composition is less open to interpretation

The Nature of Crystal Growth

Again, a question might arise That is interesting but what can one

make of it? Here something must be said about how crystals grow You have all been discussing the question of what determines the site at which the bone salt will deposit in the biological system What determines the site at which the mollusk will form its shell? Last year Bevelander[234] mentioned the point and said a little but about it He said that alkaline phosphatase was present at that position and collagen fibers were also present at the place where the shell was deposited Let me ask this question why do you run up against an exact parallel with the behavior of bone in crystal properties in a biological system which has made the same equivalent use of these properties? Well this has to do with crystal growth

Now crystal growth is a complicated subject also It is more complicated than crystal optics For instance the Faraday Society in 1949[235] devoted a full conference to the subject The reason I mention this is that the ideas about crystal growth have changed immensely within the last five years They had their foundations earlier than that but most of what we now know about crystal growth is a result of the economic importance of metallurgy It is because metals owe their characteristics so much to the physical properties that are involved in growth that it has been necessary for the metallurgist to examine the subject closely

Actually what these two biological systems have in common is the following in the mollusk shell the same condition approximately is met at one and the same time for two substances to form either aragonite or calcite Aragonite is the more soluble metastable form The same condition is met in bone where an inorganic material more or less is precipitating You have all argued deeply and long about whether there is a biological interaction or whether it is just an inorganic phenomenon In bone formation the same condition approximately is met at one time for three substances to form hydroxylapatite calcium phosphate dihydrate and calcium carbonate One condition I appreciate is somewhat apart from the others Hydroxylapatite can form as well possibly, as tricalcium phosphate dihydrate—since the conditions for the precipitation of this latter substance are not very different from those of hydroxylapatite and finally calcium carbonate In the one system the aragonite forms more often than does the calcite The classical physical chemists always laugh that off in what I have always thought even as a student was an absurd way They say that if a metastable form is likely to be present it is going to form first A better statement is that the metastable form is not likely to form but if it

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[234]Bevelander G (Discussion on Mollusks) *Trans Macy Conference on Metabolic Interrelations 3 222 (1951)*

[235]Frank F C *Discussions of the Faraday Society No 5 Crystal Growth (1949)* Gurney and Jackson Publishers London

forms it will form first It has no other alternative There is a very nice distinction between those two statements

A crystal does not grow when its solubility product is met, but rather when this product is considerably exceeded The reason is that in some way the lattice must be started After the lattice is started it will not necessarily continue to grow at saturation For instance, calcite forms a plane containing certain groups, calcium and carbonate and then stops growing because more energy is needed to begin the deposition of another plane of atoms than is required to continue a plane once it has started to form In other words unless the process involved in the initial growth of crystals includes a method for the continuation of the growth the crystals are continually cutting themselves off This shows up in mineralogy in many odd phenomena the existence of which the mineralogists realize but the causes of which they do not appreciate

Within the last three years Frank,[24] a physicist in Bristol has come up with a demonstration that most crystals have to grow by what can be called spiral dislocations A spiral dislocation arises when the repetition of these depositing groups of atoms is slightly out of phase Then the growth can follow a continuous spiral while maintaining the translational properties of the lattice and the crystal optics

The important point in these phenomena in bone apparently is that materials that are excluded appear external to the lattice between the translational elements There they constitute a disruption to the continuation of the lattice Specifically in the case of the mollusk shell the extraneous material seems to be magnesium Magnesium is excluded from the aragonite lattice and appears on the faces in the order of a few tenths of a per cent This causes the continuation of the lattice The implications are immediately evident in the case of the bone material The apatite crystal will grow only in a very adventitious position at which there is a sort of nucleation for the crystal, and it will continue to grow only because of these types of disruption of phenomena that actually are taking place in the lattice

I think that is all that needs to be said It is not a simple subject with which you are faced I would like to mention that one of the points disturbing Dr McConnell *is that I apparently have held from time* to time that the carbonate apatite does not exist I have always meant by 'it didn t exist' that the carbonate is not substituted in the lattice That the carbonate is present and that the minerals such as francolite exist, is self *evident* The argument about these minerals and bone is how the carbonate is present

Conference Discussion

Armstrong Thank you very much Dr Hendricks I shall now ask Dr McConnell if he would like to make some comments apropos of the presentation made by Dr Hendricks

McConnell I would like to make a very detailed reply to some of the questions which Dr Hendricks has raised He has referred to me as bothered or troubled by some of these points I am not sure that I am I think there is one fundamental question that arises It deals with the two-phase system chalcedony which is even better known to the mineralogist and has been shall we say more thoroughly investigated than the two phase system conchite

In chalcedony we get the same diffraction pattern as we get with quartz There are no intensity differences and no differences in the sizes of the unit cell but the optical properties as in conchite are measurably different beyond experimental error However if one looks at chalcedony it is not an optically clear homogeneous system It is a cloudy material which produces scattering of light in the visible range with no difficulty in observing that scattering at all Conchite in so far as I am aware also appears to be two phases and gives a scattering within the visible range What the other phase present may be we are not quite sure Dr Hendricks has implied that it contains magnesium I am quite willing to accept that It also probably contains some organic matter which has been produced as already outlined by these little invertebrate animals

However in francolite we have a different situation These crystals are optically clear and homogeneous Yet the unit cell edges by x ray diffraction methods are measurably different beyond experimental error and the intensities of the atomic planes which produce the interference maxima are different If I may be pardoned for saying so Dr Hendricks the differences in intensities are not due to orientation The orientation problem has been considered and a statement bearing on it will appear in a manuscript[2,6] now in the hands of an editor but I have the detailed data here if anybody wishes to see them Dr Hendricks had an opportunity to see them last summer including some unpublished charts made at the National Bureau of Standards which show differences not only in the spacings of these diffraction lines for fluorapatite and francolite but also very marked differences in the intensities Precautions were used to prevent

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McConnell D The Problem of the Carbonate Apatites IV Structural Substitutions Involving CO and OH *Bull Soc Française Minéral Cristal* 75 in press (1952)

orientation in that the samples were not compacted but were put loosely into the vessels, by Mr Howard Swanson, who is making the standard comparison diffraction data for the American Society for Testing Materials at the National Bureau of Standards

If one challenges, then the intensities on these apatite and francolite curves, he is challenging our entire system for measuring intensities Now, maybe Dr Hendricks wishes to do so but that would imply that we certainly have some increments of knowledge missing in the determination of our diffraction data because I have here the sample which was especially prepared by Mr Swanson, to determine these intensities and the intensities are different

I think nobody has denied that the cell edges are different If the cell edges are different, if the atomic scattering factors are sufficiently different to contribute these differences in intensities then I believe that the crystal structures, although remarkably similar must be different I think Dr Hendricks made a point that the refractive indices go down in francolite They go down very very little I wish I had with me the calculations that have been made on that point

Hendricks I can give them to you, if you like

McConnell Well, I have some of my own (Laughter) Dr Gruner and I first considered the applicability of the 'Lorenz Lorentz formula back in 1940 [237] I believe, and there is a good bit to be said for it However, we don t know, really, with great precision the ionic refractivities of some of these atoms in different environments

Hendricks You don't have to

McConnell Certainly, if we are considering these data with reference to the vectorial properties of crystals, that is directionwise as you indicated then we must know very accurately the ionic refractivities in order to come out with vectorial calculations The amount of water substituting for calcium, to get the spatial equivalence, if you remember, was 10 parts in 1000, or 1 per cent I am not prepared to state that we know the ionic refractivities of any of these constituents to any such precision as 1 per cent

I think there was another difficulty There was an attempt on Dr Hendricks' part to discuss bone I think we would like to discuss bone, but I don't think we are prepared to discuss bone because that is an extremely complex system I have attempted to consider the data which Hendricks

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[237] McConnell D and Gruner J W The Problem of the Carbonate Apatites III Carbonate Apatite from Magnet Cove, Arkansas *Am Mineral* 25 157 167 (1940)

and Hill have presented in the National Academy of Science Proceedings[238] and I saw that there was one notable omission They did not use the Brunauer Emmett Teller method [39] for determining surface areas on the mineral, fluorapatite I wish you had determined these surface areas We subsequently did and we got the same value for fluorapatite that we got for francolite within the error of the measurement [240] But I think it is very interesting to presume momentarily that those discontinuities actually exist in a francolite crystal such as Hendricks and Hill have done and then see what conclusions necessarily follow

One concludes without any knowledge of the type of discontinuity which occurs that these discontinuities would have spacings comparable to the wave length of light in the visible range and therefore would convert these francolite crystals presumably to beautiful spectral gratings of a three dimensional sort If you will recall there was a difference between the experimental and the calculated B E T surface areas by a factor of 9 which I did not consider particularly good agreement If one takes the other extreme namely the 60 square meters per gram of surface area and calculates for a regular system of coordinates what the average interruption of that system would be he obtains a value of 315 Angstrom units which should produce marked broadening of the x ray diffraction lines and should greatly increase the low angle scattering Nothing of that sort is observed and the diffraction lines which I showed you were not high angle lines but were fairly low angle lines I have forgotten but I think they were around 40 degrees In fact the francolite gives almost as nice diffraction lines as the fluorapatite There is a little decrease in the alpha 1 alpha 2 resolution which would be expected in terms of the isomorphic substitution which occurs

Hodge What was the surface area for the francolite?

McConnell I will have to look at the original article which Hendricks and Hill published I have it right here They have a table that gives several values For francolite below six micron particle size they show 7 3 square meters per gram and when they used somewhat coarser material 100 to 200 mesh they obtained 3 2 square meters per gram and when the particle size was 20 to 35 mesh they had 1 6 square meters per gram For the steamed bone they obtained values which approximate 66 square

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[238]Hendricks S B and Hill W L The Nature of Bone and Phosphate Rock *Proc Nat Acad Sc* 36 731 737 (1950)

[239]Brunauer S *The Adsorption of Gases and Vapors Vol 1 Physical Adsorption* Princeton Univ Press Princeton N J (1943)

[240]These unpublished measurements were made by Mr C M Hunt Mineral Products Division National Bureau of Standards

meters per gram But over on the next page they use the fluorine content as the basis for calculating the surface area of the discontinuities in francolite and obtain as I recall 61 square meters per gram I don t see the figure right here And so it depends entirely on whether you use the calculated value of 61 square meters per gram or some average measured value Actually I used the measured value of 7 3 square meters per gram which is the largest experimental value obtained On that basis one can calculate the regular periodicity of the interruption of that lattice to be about 2600 Angstroms

Hodge You mean the 2600 Angstrom length corresponds to the 7 3 square meters per gram?

McConnell Well the greater the surface area the smaller the unit of interruption of the lattice obviously so 315 Angstroms would correspond to the 61 square meters per gram and 7 3 corresponds to 2600 Angstroms But 2600 Angstroms is capable of producing scattering in the visible range and 315 Angstroms is capable of producing very significant scattering of x rays There is no evidence for either phenomenon.

Hodge But I thought you said it was consistent with the picture of bone

McConnell Well, yes it may be but the point is that I think Dr Hendricks uses the terms bone' and francolite unless I am mistaken almost as though they were interchangeable

Hendricks I have used them perfectly interchangeably

McConnell Well, thank you for doing that

Hendricks Francolite contains fluorine and that is the only distinction between francolite and bone

McConnell The surface areas are not in any way comparable though

Now I gathered that you made two attacks on my structure Is that correct? One was pertaining to this problem of the intensities being due to orientation That in no measure affects the size of the unit cell of course does it?

Hendricks No

McConnell So we still have that problem to contend with But I think I have been successful in demonstrating that these intensity measurements were obtained by the very best methods presently available to us The other attack I gathered had something to do with the general calculations that were made on crystal-optical properties and involved the decline in refractive index of the francolite below that of fluorapatite The one index

drops down but the other one goes up Let us consider the average of the data in the literature If you assume a heterogeneous system that is if you assume that there are two components you come out with almost the same refractive indices that one would obtain on the basis of that structure But this francolite crystal differs entirely from chalcedony It differs entirely from conchite in the fact that it is a single phase and not two phases and there is so far as I am aware with all due apologies to the Brunauer Emmett Teller method of determining surface areas no other evidence whatsoever of any discontinuities in these crystals On the contrary there is evidence that there are not discontinuities of the sort which have been described namely 315 Angstroms or 2600 Angstroms depending on which of these various surface area measurements are used

Armstrong This discussion certainly makes a biologist feel a little less disappointed about the progress of his own problem If you people disagree as to the constitution of a large crystal of an earth apatite what can be expected as to a definite opinion about the constitution of bone mineral? Now Dr Hendricks I believe the debate—

Hendricks Is this rebuttal?

Armstrong This is rejoinder

Hendricks How long do these discussions go on?

Armstrong We are going to have lunch in a few minutes

Hendricks Well there is no point in making a rejoinder on the change in the diffraction pattern All I have to assume at the moment now is that Dr McConnell and Dr Armstrong and Dr Gruner can take the diffraction pattern at one time and state explicitly that there is no difference The difference being you note that the *(300)* interference became the strongest interference on the diffraction pattern and in the other it was of the order of half the intensity of a more intense interference That is a thing easily picked up at any time and you all took the diffraction patterns of this It was the same francolite specimen wasn t it?

McConnell I think you will find that that statement is qualified adequately to cover these subsequent data I think it showed that there were several small differences but they were recognizable differences as I remember and that is all we have shown now really

Hendricks You have confounded the issue here somewhat however by pointing out that the atomic refractivities are not exceedingly precise You notice that I changed one for instance from 3 50 to 3 60 up there on the board That is quite precise But the point under discussion here is not involved with the precision with which those numbers are known Instead

what you did was to produce a small change in a crystal and you are concerned with the nature of that change You are concerned with the difference between two large quantities and not with the value of the absolute magnitude of either one of them That is the precise thing to look at When looked at in that way, the francolite drops

Now, I think I would like to put on the board the refractive indices of dahllite

McConnell Dr Hendricks, may we stick to francolite since that is the case under consideration? I think that would greatly simplify the problem

Hendricks Well your own values for francolite are 1 625 and 1 632 (see Table XIII)

McConnell I am glad you know them I had forgotten them myself (*Added later* The mean refractive index for fluorapatite is given by McConnell and Gruner[237] as > 1 631 but < 1 634 The values greater than 1 640 in Table XIII appear to be *from some other source*)

Hendricks And your value for hydroxylapatite or the corresponding fluorapatite is 1 640

McConnell That is a decrease of approximately seven parts in 160

TABLE XIII

Refractive Indices of Various Compounds

	α	β	γ
	Carbonates		
Aragonite	1 5301	1 6816	1 6659
	2E = 31°		
Conchite	1 523	1 659	1 662
	2E varies from 0° to 27°		
	Phosphates		
Apatite	1 6417	—	1 6461
	uniaxial		
Francolite	—1 625	—1 632	—1 632
	variably biaxial		
Tricalcium	Ca 1 59	—	—

Hendricks As a matter of fact if you take the best value for the mol refractivity it leads to a drop of mol refractivity of 2 4 It is less clear here than it is in the case of the tricalcium phosphate hydrate in which it is exceedingly clear Again it drops the refractive index and this is a question of the difference between two substances and not of the absolute magnitude That is the point to be emphasized

McConnell Would you care to say what influence the fluorine would have on that Dr Hendricks? I am sure you are familiar with the influence

Hendricks Practically none

McConnell Oh I beg your pardon! Fluorine is—

Hendricks As a matter of fact if you take dahllite the indices of refraction are 1 625 1 6?7 Now to go ahead and throw the clash absolutely in the open the case of the partial solution is not to be disposed of by the example of feldspar What you actually know in the case of feldspar is this that if you boil it with water the water gets strongly alkaline in other words there is a surface reaction that goes on which does perhaps preferentially remove the potassium who knows? But to take results like those of Cartier and say that the calcium carbonate does not completely dissolve out of that system—well it just looks to me to be pointless It just does completely dissolve out There are no other crystal systems of that type which behave like that in which you dissolve out one component and leave 85 per cent of the material there Every argument I know states that the calcium carbonate is there extraneously

McConnell On the other hand Dr Hendricks that is a very nice argument which fits with your assumption of these discontinuities Of course in the first place you call upon an entirely different theory of diffraction of crystals from any previous theories which have ever existed Unfortunately the only example of this hypothesis is the material under discussion namely francolite Now if one attempts to examine this thing in a precise way the CO_3 groups as I understand it are at the surface of the discontinuities Am I correct thus far? Please correct me as I go along if there are any errors

Hendricks That s right

McConnell These CO_3 groups have a negative charge of 2 and they must be in electrostatic balance The only conveniently available constituents to bring them into electrostatic balance are constituents of the lattice itself Presumably these constituents are the calcium ions We wouldn t want to trouble the phosphorus ions in spite of their much higher charge for a number of reasons which need not be elucidated here The calcium

and the carbonate then are related in such a way as to produce these discontinuities which you and Hill have described I am assuming that for the purpose of argument

Then how would that affect the periodicity of the lattice in the a direction? If we assume that those CO_3 groups are on cleavage planes which are perpendicular to the c axis for the purpose of argument on the front pinacoid which we call *(100)* would these CO_3 groups increase the dimensions in the a direction or decrease the dimensions in the a direction? I can hardly conceive of any mechanism by which they could decrease the a dimension and yet that is what actually transpires The francolite is smaller than the fluorapatite by 0 05 of an Angstrom

Well now let s put the CO_3 groups which are adsorbed on these discontinuities in some other place The next simplest place to put them would be on the basal pinacoid on the plane *(001)* again balancing their charges with the calcium ions that are available in the lattice itself All that is observed is an increase of 0 01 of an Angstrom unit which is quite small with respect to the size of these CO_3 groups But we are putting the CO_3 groups in the structure in a completely asymmetrical manner which is not related necessarily to the lattice of francolite, so we should not obtain any regularities in the changes of the spacing of the unit cells nor in the intensities of the diffraction maxima Nevertheless we do observe those things by actual experimental technics

I cannot refrain from repeating something that has already been said that if all of the physical properties of the francolite are different from all of the properties of the fluorapatite by small quantitative amounts the structures of these two substances must be different Then it becomes a question of speculating on what these differences could be and I have attempted to show that the speculations which I have made would account for some of the experimental differences, namely the intensities of the diffraction maxima for the prismatic zone—you will recall the intensity of the line *(300)*—should be increased and the birefringence of the crystals likewise should be increased

Bartter When you grind francolite, can you dissolve off the CO preferentially?

Hendricks Yes you increase the preferential solution up to the order of 20 per cent of the CO_2 as preferentially taken off after grinding but very little preferentially before grinding

Bartter And you believe if you ground it fine enough you could get it all off?

Hendricks I don t know Don t force me into an apparently logical

hole I could answer Yes but I also have reason to believe and I think it will come out later if Dr Hodge presents some data he has that you can not necessarily grind francolite so fine as to get at all of the carbonate Maybe you would have to grind it down to the order of 1/100th of a micron in order to do that and you cannot grind down to those dimensions

Armstrong Protessor Dallemagne you were unusually modest this morning during the debate between Dr McConnell and Dr Hendricks Perhaps you thought that two persons involved in the discussion was enough and three would make one too many I would like now to give you the opportunity to make further comments

Dallemagne If you please I would like to ask Dr Hendricks if he thinks that water adsorbed by tricalcium phosphate can explain the low value of the refractive index of that component

Hendricks No it would have to raise the refractive index

Dallemagne It would have to raise it?

Hendricks It constitutes oxygen in the volume

Dallemagne Yes but if oxygen is not in it how does it enable you to raise the refractive index? I speak about the adsorbed water

Hendricks Dr Dallemagne you can grow crystals of this so called tricalcium phosphate of the order of 2 microns in length The refractive indices are measured on those crystals Dr Melon s measurements I judge were made on crystals of that type Now if you adsorb water onto that material I can t see how you can lower the refractive index of it Such a procedure is adding oxygen or is adding the polarizability of oxygen to the system

McConnell Except that that is what happens in chalcedony isn t it Dr Hendricks? The refractive indices go down in terms of the adsorption of water

Hendricks In chalcedony I judge the refractive indices go down merely because you are measuring the refractive index of a mixed quartz air system

McConnell Yes that is right

Hendricks That is what I am holding here that when you measure the refractive index of tricalcium phosphate you are measuring the refractive index of an air apatite system and adsorption of water within that air apatite system raises the refractive index rather than lowers it It puts something other than air into it

Dallemagne Yes, it is probably the same problem, but what do you think about the raising of the refractive index of tricalcium phosphate after boiling this substance in water? The refractive index of tricalcium phosphate is raised from 1 59 to 1 61

Hendricks Well, it is losing water

Dallemagne No

Hendricks Then what is it doing?

Dallemagne I don't know

Hendricks I don't know, either

Dallemagne Generally, it contains more water

Hendricks In other words, there are two numbers given 1 59 and the later point, which I didn't fully realize at the moment 1 61 was also given Now, that 1 61 was obtained by boiling the sample I believe

Dallemagne Yes by boiling

Hendricks Well, I dont know fully what happens The only thing I would do is to use the 'Lorenz Lorentz formula and say there is less void in it

Dallemagne We don t know what happens after boiling

Hendricks That is all I could say The index went up and there is less void

Dallemagne It is the only thing we see

Hendricks Now, structurally is there any other explanation that one would suggest, other than less void?

Dallemagne Structurally the interference lines become sharper

Hendricks No, I would just say that the substance is recrystallized and in being recrystallized it is reduced to void volume

Dallemagne But what do you propose about the structure, the molecular structure, of tricalcium phosphate?

Hendricks Oh, I don t know I can t give a convincing argument as to what that structure is You get me on thin ice if you ask me to draw the pattern of it but I'll get up and skate once (Laughter) Maybe I have an illustration that might show it better and that will save time

Figure 36 shows the surface of what these things are and it tries to

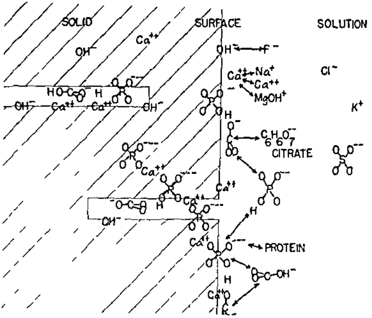


Fig 36 Schematic Indications of Surface Compound Formation in the Bone Salt

explain several of the points that have been somewhat hidden in the arguments Instead of answering Professor Dallemagne directly I will answer him after another fashion I think Dr Dallemagne has argued that in enamel the carbonate is present as carbonate apatite and in bone, the carbonate is not there as apatite The reason he has so argued is that upon heating to something like 1000°C, the carbonate of the two substances behaves differently In the case of bone, it is lost, and in the case of enamel, it is not lost

Dallemagne And another argument is that the refractive index of enamel is much higher than that of bone

Hendricks Yes, it is much higher Possibly the void system in enamel if this void argument is correct, is buried within the crystal and the carbonate cannot escape from it upon heating The calcium to phosphorus ratio has to be reduced to make tricalcium phosphate, which requires addition of phosphate to the system The argument based on refractive index indicated that phosphate was removed from the system If the volume remained the same, phosphate would have to be added to the system in order to raise the calcium to phosphorus ratio to 3 2

Tricalcium phosphate hydrate is prepared at about pH 5 The predominant molecular species in the solution is $H(PO_4)^{=}$ and this or $H_2(PO_4)^{-}$ goes onto the surface The question now is the behavior of hydroxylapatite in exceedingly small crystals with $H(PO_4)^{=}$ ions or with $H_2(PO_4)^{-}$ ions on the surface I can well imagine that such a lattice would be extremely permeable to hydrogen ions that they could wander in quite readily and give the unusual water loss properties of the hydrate This hydrate is indeed unusual in the nature of its water loss and contrasts with most hydrates by being zeolite like Perhaps the migration of the hydrogen ion from the surface into the crystal really takes place

To answer specifically about the acid phosphate I would say that it can be on the surface and the hydrogen ion can migrate relatively freely through the lattice as a whole because it is essentially an ion without size As a matter of fact it could be the reason for loss of water from hydroxylapatite at 600° rather than having to go to 1300° as with normal hydroxylapatite According to this idea the way to dehydroxylate hydroxylapatite would be to put acid phosphate on its surface and then heat the material

Dallemagne I think that if dicalcium phosphate were on the surface the measure of the refractive index would give the refractive index of the dicalcium phosphate

Hendricks Oh no it does not

Dallemagne Not necessarily?

Hendricks This is not a surface with tricalcium phosphate or dicalcium phosphate on it The refractive index of a material depends upon the presence of voids and surface components and is determined by the content per unit volume

Dallemagne Isn t it true though that the conditions of precipitation of hydroxylphosphate are not the same as for dicalcium phosphate

Hendricks Yes that s right

McConnell Mr Chairman I have said nothing about Dr Dallemagne s presentation and indeed I have very little to say about it because he is considering quite a different aspect of the matter than I am I think we can simplify many of these mineralogical arguments as long as we confine ourselves to minerals When we get over into these organic inorganic systems the situation certainly becomes extremely complex as I believe all of us will fully agree

But I think it is feasible irrespective of the refractive indices to consider the hydration properties of this so called alpha tricalcium phosphate',

by virtue of the fact that if we write our conventional formula *with the fluorine* as we familiarly do it is not impossible to arrive at something which would have ratios of calcium to phosphorus which would be comparable to those that Professor Dallemagne suggests But we don t come about it by any such simple technics as Professor Dallemagne suggests according to my concepts

I have *postulated then for this tricalcium phosphate hydrate the alpha* form that there are hydroxyls in the fluorine position and that this compound is a normal hydroxylapatite except that it has water substituting for calcium and hydrogens substituting for phosphorus Now it is experimentally a fact that if you vary the environment of the precipitation of this alpha tricalcium phosphate the ratio does not come out precisely 3 2 and this phenomenon isomorphic substitution will very nicely explain departures from these atom ratios This phenomenon will explain the deviations in exactly the same way as I have called upon it to explain the departures in francolite

Now with respect to the steamed bone and to the undeproteinized bone when we have protein present we have no hope for allocating appropriate amounts of water to three structural positions in such compounds as long as we have still another fourth type of water involved in the protein substance or collagen or whatever one wishes to call it In fact the analytical difficulties here are almost insurmountable because we don t know the energies of bonding of these hydroxyls and water molecules in the alpha tricalcium phosphate

I had hoped to avoid a discussion of the mineral viseite which I recently received from one of Professor Dallemagne s colleagues *Melon but it is* a zeolitic type substance It has a very complex structure which is nevertheless cubic It is both a phosphate and a silicate but in this instance large portions of the structural framework the YO_4 tetrahedra *be they* phosphate or be they silicate are substituted by hydrogen atoms to the extent of a third of all of the XO_4 groups in this entire framework structure containing hydrogen rather than silicon or phosphorus This morning I gave you another analogy—the hydrogarnets in which all of the silicon is substituted by hydrogens

Armstrong Are there any further comments or questions?

Dallemagne One more please In your hypothesis of the hydroxylapatite as the base of the compound the dicalcium phosphate is on the surface of the crystallite?

Hendricks No not dicalcium phosphate *Professor* Dallemagne but sufficient phosphate as to make the sum of the formula that of tricalcium phos

phate Dicalcium phosphate is not on the surface but enough hydrogen is there to balance the charge so as to make it tricalcium phosphate and that will turn out to have the amount of water the H_2O for the Ca_9 or Ca_{10}

Dallemagne And we must explain the transformation of that compound at 700°C into the dehydrated tricalcium phosphate

Hendricks Yes, and if you try to pursue that in detail you are forced to the conclusion that the hydrogen migrates into the lattice combines with the hydroxyl and is lost in water You are rather forced into that structural conclusion

Dallemagne But the hydroxylapatite is stable at 1300°

Hendricks Yes and that was the point about it Is it stable in the presence of surface hydrogen at 1300°C ? Is not this the way to make it unstable at 700°C ? Pure hydroxylapatite is an interesting substance because it goes to 1300°C without losing its water The reason possibly is connected with the great separation of hydroxyl groups Now if hydrogen migrates through the lattice, it can form water with the hydroxyl The argument might not be worth anything but it would be a tenable sort of argument until other facts are presented against it

Hodge This is not exactly on the present point Mr Chairman but I would like to make a plea a plea you have heard before for the abandonment of the term *'alpha* in connection with this precipitated material The plea which I have made here before is based on the fact that there has been for a long time, an alpha tricalcium phosphate in the literature When a molten mixture of CaO and P_2O_5 in amounts such as to approximate tricalcium phosphate is allowed to cool the first crystals to separate out are called *alpha tricalcium phosphate'*

These crystals are well defined crystals with a characteristic x ray diffraction pattern and with optical properties that have been measured by several people (most recently by Bonner[241]) There is a secondary change in this material somewhere around 1350° C —I forget the exact figure— with a modification in the crystal structure a new crystal form appears and this *beta tricalcium phosphate* also has a characteristic x ray diffraction pattern and characteristic optical properties The first crystal that separates out from such a salt is usually referred to or frequently referred to, as the *'alpha form* The form that appears at lower temperatures is frequently referred to as the *'beta form* These two terms applied to tricalcium phosphate have been in the literature quite a while, so that *alpha*

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[241] Bonner J F, Jr *An Investigation of the System CaO P O*, Th Rochester, Rochester New York (1940)

tricalcium phosphate is by definition the crystal form that first separates from the melt

If you start with the equivalent of tricalcium phosphate by mixing CaO and P_2O_5 and warm this mixture in a crucible the first crystal that develops is the *beta form* and with increasing temperature it changes without melting into the *alpha form* again Just for the sake of clarity in nomenclature I wish we would not call the precipitate from water solution *alpha* tricalcium phosphate hydrate because the precipitate does not have the x ray diffraction pattern or the optical properties of the first crystal that separates from the melt

McConnell That same confusion applies however also to quartz Dr Hodge to the extent that we now refer to *high* and *low* quartz rather than *alpha* and *beta* because the two opposing nomenclatures exist in complete parallel This is not a unique situation in which we use exactly the same two Greek letters to mean exactly opposite things Quartz is the most common example

Armstrong I am sure that biologists will not be confused by these differences in terminology Dr Hendricks has another figure for us but before he discusses it I want to flash back to the one he just presented (Figure 36) to ask him a question When you allow the adsorption—or perhaps I had better use the word *association*—of ions at discontinuities of the crystal surface do you require that there be an equivalent number of positively and negatively charged ions?

Hendricks Yes of course at any rate in the system as a whole

Armstrong Well if that is true then one should be able from the analysis of the material to calculate the number of ions due to the hydroxylapatite component and find a cation—anion balance among the ions not relegated to hydroxylapatite

Hendricks Oh yes and we do precisely this but it doesn t help It is just balancing the books They will balance either with the modified crystal structure or with the phosphate and other ions on the surface There is no difficulty about that at all In other words calcium is associated with the negative surface ions There is calcium at the edge of the crystal and you don t know how it stops

Armstrong You referred to hydrogen ion supplying some of the positive charges

Hendricks Yes the hydrogen is indicated Figure 36 is just schematic and doesn t prove anything It just helps you focus this point of view in your mind

Armstrong In the case of calcifications occurring in biological media do you think the concentration of hydrogen ions would be sufficient to add any appreciable number to the product?

Hendricks I know the pH system is around 7 There are two answers "Yes," and "Possibly so" I don't fully know though

McConnell I would like to comment on the status of this material which might according to Dr Hendricks thesis be adsorbed on the surfaces If it is the carbonate constituent the most stable form in which it could conceivably occur would be the calcium salt calcium carbonate in which case it should not be retained at any such temperatures as 1100° If it is present as H_2CO_3 it should leave very easily at much lower temperatures Actually, if you take some of this mineral francolite you can heat it to approximately 1100°, and hold it there for some considerable number of minutes—I think 45 is the longest period of time I have held it at that temperature—and you analyze the material and find that it still contains CO

Now, I can't conceive of any bond linkages there which would be more stable if the CO_2 is actually not in the apatite lattice than the bonds in calcite and of course, the dissociation temperature of the calcium carbonate is far below 1100° It is if I recall correctly in the vicinity of 800°C

Hendricks Well, there are various things one could say in answer to that One of the simplest ones is that Dr McConnell is a silicate chemist and he is familiar with the fact that to dehydrate silicate melts it is adequate to melt and crystallize them You can very easily get silicate melts to retain water at 1400° Another common case is that quartz crystals often contain water, and you can heat them up to 400, 500 or 600°

About the carbonate dissociation I would say that the carbonate in the surface compounds really has a tendency to break up and give off CO at temperatures on the order of 600°, which is the temperature at which bone—that is not too much involved with entrapping the carbonate—will permit it to escape

Armstrong Would you anticipate that hydroxylapatite synthesized in—I believe the word is "different environments"—would have a constitution or composition of extraneous materials which would be closely related to the composition of fluid from which the precipitate is formed?

Hendricks Yes, certainly To answer that other question about what is going to come down in the case of bone you don't escape it You devise a structure, and analyze it, and even if you find that it comes out the composition of tricalcium phosphate you in no sense escape the difficulties of

your pH argument Irrespective of that system you still, apparently, would have to have hydrogen ions there that you normally would not associate with a pH of 7 Do you see the point?

Armstrong Yes

Hendricks No matter what the argument is as to how you put the stuff together, if it comes out at pH 7 and is tricalcium phosphate, you still have to argue that it apparently has hydrogen ions present

DEHYDRATION STUDIES WITH SYNTHETIC HYDROXYLAPATITE

WILLIAM F NEUMAN

From the Division of Pharmacology and Toxicology Department of Radiation Biology, School of Medicine and Dentistry University of Rochester, Rochester, New York

Neuman I want to add one more note to the surface idea Figure 37 is derived from some studies with synthetic hydroxylapatite We were trying to separate the apatite from solutions of varying composition and of course, as a control, we had to make sure that we had only solids with none of the contaminating solution

We were very surprised to find that the water did not come off those crystals very easily I was assured by an analytical chemist, who had been studying a number of hydrated compounds, that centrifugation at 2000 r p m in a sintered glass filter would get rid of mechanically held water We centrif[illegible] at 2000 [illegible] 200 m[illegible] per[illegible]

cent of its own weight of water

There was another check on this in which we labeled 'surface phosphate with P^{32} and followed that by centrifugation of 40 000 times gravity We know that the water, if it were just contaminating water would contain P^{32}, and therefore roughly, 40 per cent of our P^{32} should come off Instead we removed only a few per cent

I am now quite convinced at least at the moment, until more data come in and I change my mind (laughter), that what we have in this system is a crystal which has a charge probably a negative charge Surrounding that is a hydration layer in which you can have a host of different ions reflecting the solution properties and the solution composition

Conference Discussion

Armstrong One other point that has not been discussed today—perhaps it is raising a ghost that ought to remain laid—is in regard to the former teaching that calcium carbonate might exist as such in the bone mineral but could not be demonstrated by x ray spectrograms because of lack of sensitivity of the methods I believe that modern methods will disclose the presence of the calculated amount of calcium carbonate in bone if the total CO_2 occurs as carbonate In fact, two years ago, Professor Brasseur

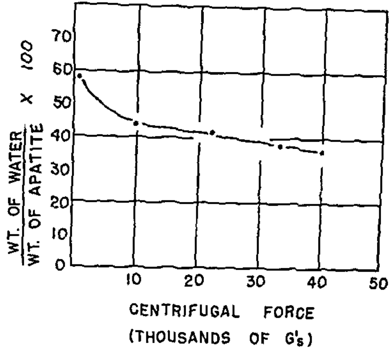


Fig 37 Evidence for the Existence of an Hydration Layer

The commercial hydroxylapatite was shaken with water and centrifuged in a sintered glass filter *In the figure are plotted the amounts of water retained at varying speeds of centrifugation*

showed me in Professor Dallemagne s laboratory, an x ray spectrogram of bone which I was willing to admit showed the presence of calcium carbonate, and you, Dr Hastings, had a similar spectrogram here a year ago Does this subject need any further comment?

Hastings I just want to say that we have been unable to get any more on ours It may have been an artefact

Hendricks I want to confuse the issue You know, you can always escape those arguments by saying the material is amorphous All the crystal structure people raise their hands in horror and say, "*No there is no amorphous calcium carbonate!* Well, in a recent number of *Science*,[21] the amorphous calcium carbonate was described But crystalline calcium carbonate can be detected, and if amorphous calcium carbonate is there, it'll

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[21] Odum, H T Nudibranch Spicules Made of Amorphous Calcium Carbonate, *Science* 114 395 (1951)

ways has to be amorphous and not crystalline at least in so far as any examination has been carried out

I would say that all of the people here today while they may have argued about many things have agreed that the carbonate is there as something other than calcium carbonate, in other words we all say I think as far as we are concerned the question is pretty well settled Wouldn t that be right? Let s agree on one point anyway

McConnell I agree to that 100 per cent (Laughter) In fact I would go further than that and say that in these mineral constituents which have some time consequence in geological age nothing has crystallized although it should certainly do so because the less energetic form would be the more stable form and that would be calcite

Hendricks And of course always the stronger argument is that the vital system is unsaturated with respect to calcium carbonate

McConnell Furthermore another point should be emphasized Whether the calcium carbonate is crystalline or whether it is amorphous one cannot deduct from the composition of francolite molar equivalents of CO_2 and CaO and come up with the stoichiometry of fluorapatite It can t be done and the discrepancies significantly exceed the analytical errors for such determinations—which is another rather straightforward argument I think that francolite is a single phase

Geiger [33] in Switzerland has recently published some data on this point He didn t arrive at any straightforward conclusions in fact one might glean from his paper that there is no solution to this problem which would be an unhappy thought at this juncture However he did show the calculated results

Follis Dr Hendricks in Figure 36 I saw the word protein (Laughter)

Hendricks That was just thrown in there for good luck

Follis I would like to bring together perhaps what we discussed yesterday and what we have heard today What is your concept of the combination of the crystal and the matrix?

Hendricks Oh I haven t any concept I haven t any good idea

Follis Is it a chemical combination?

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[33] Geiger T Contributions to the Problem of Carbonate Apatite *Schweiz Mineralog Petrog Mitt* 30 161 181 (1950)

Hendricks Yes, chemical in the same sense that all of these surface compounds are chemical They have to be If the surface concept of Figure 36 is tenable, it is a chemical combination It is a surface salt This carbonate group is negative, and there is a positive ion in here, that is just as good as if you were making calcium carbonate If that is the case, since the protein carries both positive and negative ions, and since the surface has positive groups in it like calcium and negative groups like phosphate, then the combination of the surface with the protein would be a tenable sort of thing, in so far as lysine, arginine, glutamine, and succinate and so forth exist in the protein Succinate and glutamate and substances like that that have free carboxyl, free amino acid and free basic groups Then this would be a reasonable sort of system to form surface compounds

You would expect these phenomena, irrespective of the validity of the argument about the carbonate, because ultimately the crystal has to have a surface In Figure 38 I have shown what the crystal is doing from one point of view, that is from one view of the hydroxylapatite pattern There is a regular pattern, but at the edge of the crystal, the pattern has to stop and when it stops, it cannot remain neutral and it has to have surface phenomena Something odd has to happen at the edge

One is saying that these crystals are so small that the edge phenomena have come to predominate, but no matter what size they are, they have the chance of combining with other positive and negative groups, and it might be that therein lies the key to the question of where bone precipitates

Armstrong It is a template?

Hendricks Well, I don't know whether or not it is a template, but it could be where the nucleating phenomenon lies

Kochakian Would you say that this is the growing point, that is, the centers are anchor points and further growth is due to accretion of the mineral crystals, or does the protein have to continue to grow in order to provide new centers for further accretion of the mineral crystals?

Hendricks You might have to do both There are two phenomena to meet, and this, I am sure, will be agreed to by Dr McConnell, too First, you have to start the crystal growing, which takes a nucleation center For instance, if you want to freeze water, the temperature generally has to be below zero Some sort of nucleation center has to be present and as far as we know at the moment, their centers are just conveniently called "motes" That is the one phenomenon you have to have for crystal growth The crystal starts growing, and it flips back down to less supersaturation But you have to maintain a definite supersaturation to keep it growing It won't come right back down to zero supersaturation, as a rule

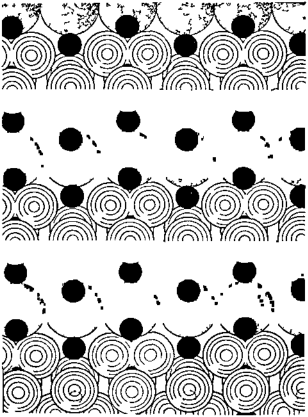


Fig 38 Atomic Arrangement on the (10·0) Face of Hydroxylapatite

The ringed spheres represent the oxygen atoms of the phosphate groups *the stippled spheres* the hydroxyl groups and *the small black spheres* the calcium ions

Kochakian What I had in mind was for example if you had a point which was one of the points on your protein molecule and you got the crystal started on that is that all that is necessary? Would it just keep on growing or would it go let us say to a certain size and then you would have to start another spot with more protein?

Choucroun Yes

Hendricks It could be either way

Johnson That gets to another question I would like to ask Dr Hendricks Since the growth of a crystal of which you gave us some inkling

earlier this morning is a matter of displacements and since the growth of mineral crystals such as francolite ordinarily produces massive crystals compared with those found in bone is there any way of approaching mathematically the calculation of the possible role of protein in decreasing the amount of displacement or decreasing the duration of displacement and hence being the limiting factor for crystal growth so that it cannot reach the size in some of your minerals?

The statement is made that colloid systems modify crystal growth producing abnormal growth along certain axes or limiting growth along other axes as contrasted to inorganic mineral systems I wonder if perhaps we can approach it some way here through the displacement idea and relate our protein problem to the crystal problem a little bit

Hendricks Well we know a lot about these things but not enough to make a quantitative calculation on any crystal growth phenomenon practically speaking

McConnell Even the most favorable situation which is dental enamel is not clear We are still not in any position to do that even for dental enamel There the individual crystals are this carbonate apatite—if I may be pardoned for calling it that (Laughter) We run into textural and structural difficulties The fabric of tooth enamel is very simple compared with what it is in bone because the continuity of the apatite crystals be they carbonate apatite or hydroxylapatite or whatever they are has some discrete dimensions But the crystals that we are talking about in bone are extremely small and give these very wide lines and very diffuse diffraction patterns because of the extreme smallness of the crystallites, so one has to approach this question I believe in terms of the large homogeneous crystal systems and then see what modifying factors must obtain as we encounter smaller and smaller crystallites

Now I have forgotten what dimensions we calculated but Gruner and Armstrong and I[24] gave on the basis of the measurements that we made on dentin material an approximation to the size of the crystallites and they are extremely small The periodicities of these crystallites are not continuous for any large distance and therefore they produce these very very poorly defined diffraction patterns

It seems to me that the way to approach this question is to attack the simple problems first and by all odds the simplest of all the simple problems is the question of the mineral composition of francolite If we ever

[24]Gruner J W McConnell D and Armstrong W D The Relationship Between Crystal Structure and Chemical Composition of Enamel and Dentin *J Biol Chem* 121 771 781 (1937)

solve that maybe we should go to dahllite and if we ever solve dahllite maybe we should go to dental enamel and if we ever solve dental enamel then we should start to think about bone

Armstrong Dr Hendricks I continue to return to one of your illustrations (Figure 36) I hope you will allow this figure to be reproduced in the minutes You show the association of negatively charged ions with the surface of the material—

Hendricks And positive ions

Armstrong And positive ions yes Now under these conditions would you expect it to be possible to substitute one negative ion for another? That is could you wash off the carbonate with a neutral acetate solution or a neutral sulfate solution?

Neuman You could wash it off with water

Hendricks I am afraid in many of these preparations we wash off a lot of the surface carbonate That becomes involved in the argument of how you prepare material We steam bone We treat it with ethylene glycol in KOH and glycerin in KOH and it probably does quite bad things to the surface constituents

Armstrong But let s take a mineral let s take francolite where you don t have to treat the specimen badly

Hendricks But you do You see my whole argument about francolite is that francolite pulled a joke on you It buried the carbonate and you can t get at the buried carbonate Therefore you accept an impure substance—impure in the sense of a simple substance—whereas in bone you say ' Well the carbonate can be washed off In francolite the argument is that the carbonate is so trapped that it will not dissolve without complete solution of the crystal It is an old principle I believe of industrial organic chemistry that if you want to prepare a pure substance you prepare it in finely divided crystals and not in coarse crystals The bigger you make your crystals the more impure is your material as a rule because it occludes impurities Everything goes along much worse in a big crystal than it does in a small crystal

Now Dr Hodge and I both tried to settle this by doing carbonate exchange and I don t believe that either one of us should make his figures public here at this time Don t you think that would be best?

Hodge Yes

Hendricks But the solution might lie there Dr Armstrong You said two years ago that a student of yours was working on carbonate exchange

Armstrong He worked in a system in which there were two or three labeled ions

Hendricks And he got in a mess

Armstrong I could never make any definite interpretation of the results

Hendricks Well then quite a bit more experimental data will come out on this subject when quite a bit of good exchange work is done with carbonate on bone How it will come out at the moment I don't know I did it one way and it didn't come out very nicely, and Dr Hodge did it another way and it came out quite differently, so there is no use in discussing it today

Dallemagne About bone carbonate, it is very difficult to separate bone carbonate from phosphate by means of acid, because some phosphate passes into solution

Hendricks Oh yes surely

Dallemagne You remember the experiences of Logan and Taylor [245] These authors treated bone salts with boiling N/10 hydrochloric acid and the substance lost I think 50 per cent of its carbonate content and only 10 per cent of the phosphate, the acid solution contained magnesium sodium and a small amount of calcium According to Logan and Taylor, these elements are combined in bone with CO But I think that this conclusion is not fully acceptable, because a part of the phosphorus goes into solution

I have tried to find another method to separate the carbonates from the phosphates of bone and we may operate by the following one The bone salts are prepared by Gabriel s method [246] A sample of bone salts for instance one gram is heated at 600°C for 24 hours and after this treatment, it is immersed in CO_2 free water (250 cc) The pH of the water rises up to 12 and the phenolphthalein immediately gives a dark red reaction This alkalinity may be neutralized by hydrochloric acid and this gives a first indication about the elements extracted from bone salts After complete extraction of the soluble compound the remaining solid fraction may be separated from the liquid fraction This latter contains magnesium sodium and calcium Actually, you can find in the solution all of the magnesium and all of the sodium of the bone and a small part of the calcium, but the presence of phosphorus is not detectable

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[245] Logan M A and Taylor H L Solubility of Bone Salt III Partial Solution of Bone and Carbonate Containing Calcium Phosphate Precipitates *J Biol Chem* 125 391-397 (1938)

[246] Gabriel S Chemical Studies on the Minerals of Bones and Teeth *Z physiol Chem* 18 257 (1894)

Moreover it is possible to repeat the same experiment weighing several samples of bone salts treating one of them at each of the following temperatures 100° 200° 300° 400° 500° 600° 700° 800° and 900°C After the immersion of each sample in CO_2 free water one obtains for the different temperatures of treatment the CO_2 loss of the solid phase the alkalinity and the quantity of magnesium sodium and calcium present in the liquid phase The results of such experiments are interesting They are still in progress and are not yet finished but I can tell you something about them

The substances treated at 900°C no longer contain CO_2 The quantity of hydrochloric acid necessary to obtain neutrality of the liquid phase is variable and depends on the temperature of the treatment You can see in Figure 39 that after a rise the curve drops The quantity of magnesium calcium and sodium found in the different liquid phases corresponding to treatment at different temperatures varies in about the same way

During the treatment at 600°C and over the calcium and the magnesium carbonates lose CO_2 and get into solution as oxides during the subsequent immersion in water How do you explain that CO_2 of bone is in a lattice of carbonate apatite or a compound of the same type if CO_2 by heating can escape completely from the bone? Indeed carbonate apatite is thermostable up to 900°C and more

The bone salts treated at 800° and 900°C lose less calcium and magnesium during their immersion in water because these elements are partly combined with the phosphate fraction of the bone when the temperature reaches 700°C At 900°C the formation of hydroxylapatite is more complete the second part of this molecule contains either calcium magnesium or sodium I think that this is a supplementary argument in favor of our hypothesis that is to say the possibility of the formation of hydroxylapatite from tricalcium phosphate and carbonates of bone transformed into oxides by heating

How can you explain the formation of hydroxylapatite at 700°C or at a higher temperature if the substance is primitively hydroxylapatite? Were this true I couldn't understand the fact that the residual calcium and the magnesium are able to combine with the phosphate during the treatment in the oven

A valid objection to our hypothesis would be that as we know sodium carbonate is stable at high temperature If sodium is present in bone in the form of carbonate it could be difficult to explain the fact that heating induces the destruction of this compound and the escaping of all of the CO_2 of the bone But I think that at 600°C calcium carbonate is decomposed and gives calcium oxide In the solid state calcium oxide is able to react

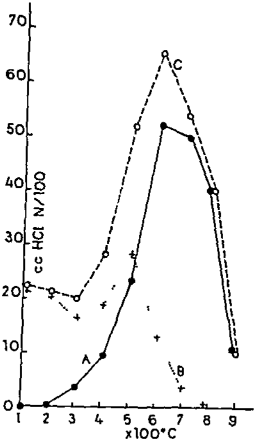


Fig 39 Titration Curves of Bone Salts

Curve A the first step of the titration at 20° C with the end point phenolphthalein. *Curve B* the second step of the titration at 100° C *Curve C* the total amount of HCl used

with sodium carbonate, through exchange the CO_3 group is transferred from Na_2CO_3 to CaO, but $CaCO_3$ is immediately decomposed and CO_2 escapes I think it is a possible explanation of the facts observed

McConnell This seems to be a question that I am called upon to answer I think the statement that I made this morning is quite apropos and possibly should be reiterated There is according to my concept Professor Dallemagne no such thing really as an equilibrium between the liquid and the solid phase in any of these dissolution phenomena There can't be of necessity in other words all of these dissolution phenomena have been produced by one mechanism or another under non equilibrium conditions There are vast quantities of these dissolution data in the literature which are quite meaningless with respect to equilibrium conditions because equilibrium cannot obtain between the solid phase and the liquid phase since the constituents are dissolving out of the solid phase at different velocities In other words there is not one reaction going on It is not a question of sodium chloride dissociating into X number of chlorine atoms and X number of sodium atoms with the same velocity, it is a question of the calcium coming out at a different velocity from the CO_4 and the CO_3 coming out at a different velocity from the phosphorus Thus as long as there is any solid phase present there will never be equilibrium in so far as we mean by equilibrium that the stoichiometry of the liquid phase is the same as the stoichiometry of the solid phase There will never be any equilibrium between the aqueous phase and the solid phase and that is exactly the sort of thing that we encounter in the case of feldspar, the example I gave this morning

I should probably give the reference to these data on feldspar The paper is by L C Armstrong and it is in the *American Mineralogist* [247] If he takes feldspar and puts it in water—and Dr Hendricks is very familiar with this phenomenon I am sure because he has alluded to it on several occasions—he immediately gets a high alkalinity which indicates that sodium has come out of the crystallites and has hydrolyzed to produce if you wish sodium hydroxide Now one can continue to extract the sodium and the sodium comes out at a very high velocity relative to the velocities of dissolution of the aluminum and of the silicon But if that procedure is carried to ultimate completion all of the constituents can be electrodialyzed and will go to the cathode, in fact you get ionic silicon apparently, by such a technic as this Don't ask me why because that is very alarming from the standpoint of silicate chemistry But the constituents do all come

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[247] Armstrong L C Decomposition and Alteration of Feldspars and Spodumene by Water *Amer Mineral* 25 810 820 (1940)

out, and the stoichiometry of the liquid phase becomes that of the solid phase but *only after all of the solid has been destroyed*

Now there is no conceivable technic—and this is a generalization which is applicable to all silicate chemistry—by which you can prepare an aqueous phase of calcium, of aluminum, or of silicon, and by any straightforward technic evaporate it or reduce the solvent content to impotency, and get a single crystalline phase which will have the stoichiometry of the liquid phase It just doesn't work that way

Dallemagne But here it is not a question of equilibrium I am extracting all that I can from the bone, because the carbonates, during the heating to 600 or 700°, are transformed into oxides and so go into solution I am extracting with hydrochloric acid with an equilibrium of pH 7—

McConnell May I interrupt you for a second, Professor Dallemagne? You say that the calcium oxide, the magnesium oxide, and the sodium oxide are present as separate phases? I am sorry, I didn't understand

Hendricks He just says they are not in the lattice

McConnell Oh they are not in the lattice!

Dallemagne They are not necessarily in the lattice

Hendricks And beyond that, there is no necessary statement

Dallemagne They are out of the lattice

McConnell Out of the lattice? Then the lattice is broken down to yield these constituents

Dallemagne It is not broken down My phosphate is not broken down during the treatment In my solution I don t find traces of phosphorus

McConnell May I ask another question? You say these are non equilibrium conditions Therefore they have some time sequence and they will change during a period of time Is that correct?

Dallemagne No

McConnell Well then why are they not equilibrium conditions?

Hendricks They are not reversible You can t put CO_2 back into the system and go back to the same system

McConnell Well in that case, there is no equilibrium either

Hendricks That is the specific reason that they are not in equilibrium

Dallemagne It is a matter of extraction, not equilibrium

Hendricks May I say something Dr Dallemagne? Dr Dallemagne has put a two pointed argument here First he has argued that because the CO_2 is lost in this way the lattice does not have the carbonate in it But second he has argued that because the carbonate is taken by heating with formation of tricalcium phosphate the lattice could not have been hydroxylapatite, therefore it had to be tricalcium phosphate hydrate

McConnell I can see that this bull has two horns

Hendricks It also has a tail (Laughter) But that is the argument

McConnell I wish we could stick to francolite It would be simpler (Laughter)

Hendricks Let somebody else have the floor Mr Chairman

EXPERIMENTAL DATA ON THE SOLUBILITY OF HYDROXYLAPATITE, ESPECIALLY THAT OF THE DENTAL ENAMEL[248, 249]

YNGVE ERICSSON

From the Department of Chemistry of the Royal Veterinary and Agricultural College Copenhagen Denmark and from the Department of Operative Dentistry of the State Dental College Stockholm Sweden

Armstrong Gentlemen I know that there are other comments which would be of interest to this discussion However I would now like to call on Dr Yngve Ericsson who has just arrived in this country from Sweden Dr Ericsson is a worker in the Dental School of Stockholm He has contributed to this subject particularly with reference to the effect of CO tension on the solubility of synthetic apatites and the naturally occurring biological apatites Professor Ericsson will you proceed?

Ericsson First I must apologize for my inability to be as free and informal as you have been gentlemen, when I am going to present a few results which might be of interest in this connection If I should try to be quite informal I would probably only be unclear

Six years ago when I was preparing an investigation of the salivary calcium phosphate with special regard to enamel dissolution and caries I came in contact with Bodil Schmidt Nielsen who was just about to finish a thesis on the saturation of the saliva with hydroxylapatite[250] She used Bjerrum s formulas for the solubility of hydroxylapatite,[251] and found the saliva as a rule strongly supersaturated, often eight or ten times supersaturated I soon found reason to doubt that Bjerrum's values really could be applied directly to the saliva and my theoretical calculations, as well as my first experimental series seemed to strengthen that opinion These were equilibration tests with saliva and powdered dental enamel at body

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[248] Ericsson Y Enamel Apatite Solubility Investigations into the Calcium Phosphate Equilibrium between Enamel and Saliva and Its Relation to Dental Caries *Acta Odont Scandinav* 8 Suppl 3 139 pp (1949)

[249] This work was supported by the Swedish Medical Research Council through its Sub committee for Caries Research

[250] Schmidt Nielsen B The Solubility of Tooth Substance in Relation to the Composition of Saliva *Acta Odont Scandina* 7 Suppl. 2 88 pp (1946)

[251] Bjerrum N Tovborg Jensen A and Damsgaard Sorensen A Investigations on the Solubility of Calcium Phosphates in Bjerrum N *Selected Papers* Munksgaard Copenhagen (1949)

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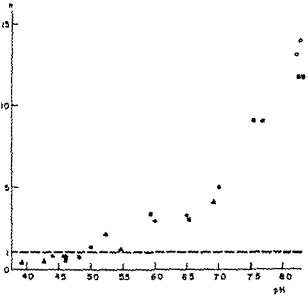


Fig 40 The Solubility of Enamel Apatite in Saliva

The ordinate gives the values for *n* which equals *the solubility found* divided by *the solubility expected from the formulae of Bjerrum*[231] the abscissa *the pH* *The open circles* are the values obtained when oc acid was added to the saliva *the solid squares* those obtained when HCl was added to the saliva *the solid triangles* those obtained when lactic acid was added to the saliva and *the solid circles* are the values obtained by recalculation from Fosdick and Starke[232]

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[232] Fosdick L. S and Starke A C Jr Solubility of Tooth Enamel in Saliva at Various pH Levels *J Dent Res* 18 417 430 (1939)

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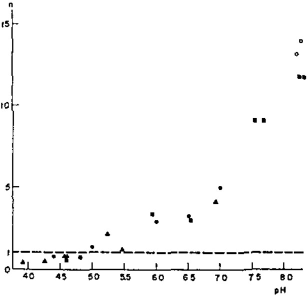


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[252]Fosdick L S and Starke A C Jr Solubility of Tooth Enamel in Saliva at Various pH Levels *J Dent Res* 18 417–430 (1939)

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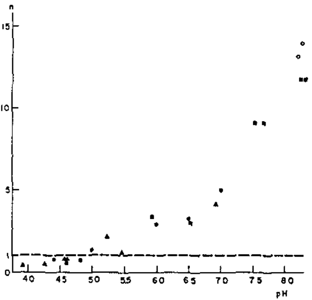


Fig 40 The Solubility of Enamel Apatite in Saliva

[2] Fosdick L S and Starke A C Jr Solubility of Tooth Enamel in Saliva at Various pH Levels, *J Dent Res* 18 417–430 (1939)

Equilibration Tests with Saliva and Powdered Dental Enamel

The results of these equilibrium *experiments* with saliva and powdered dental enamel showed especially at pH values about neutrality strong deviations from Bjerrum's figures In Figure 40 we have the pH axis along the abscissa and along *the ordinate* the relative solubility that is the ratio (solubility found) (solubility expected from Bjerrum's formula) *If agreement* with Bjerrum's formula should have been found we would of *course* have found all the points located along the broken line where the *relative* solubility is unity but as you see they seem to follow a *curved line* to the top at the highest pH values The deviations were thus of a *regular* nature and I think that is best illustrated by the logarithmic diagram in Figure 41

In this figure along the abscissa we have still the pH values of the salivas equilibrated with powdered enamel Along the ordinate we have the

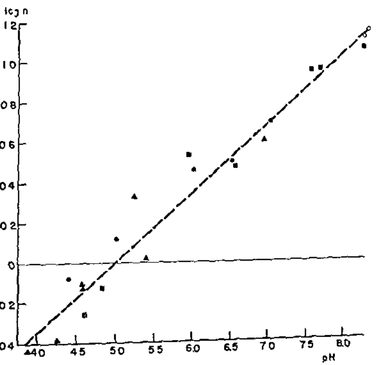


Fig 41 The Solubility of Enamel Apatite in Saliva (Log *n* Values)

The ordinate gives the values for *log n* the abscissa the *pH* The key to the symbols is the same as in Figure 40

logarithm of the relative solubility that is the logarithm of the ratio (solubility found) (solubility expected from Bjerrum s values) The experimental results are the same as in the previous diagram but in this wholly logarithmic diagram the points fall along the broken line which is a line of reference which will occur in the following diagrams too Especially at pH values of about 7 and higher we found greatly increased solubility in the saliva in relation to Bjerrum s solubility figures for pure salt solutions

Equilibration Tests with Hydroxylapatite

In control experiments with pure well-crystallized hydroxylapatite as I still prefer to call it in HCl solutions using the same method as Bjerrum and his collaborators had used in their basic experiments solubilities were

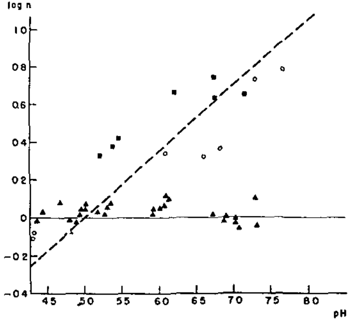


Fig 42 The Solubility of Synthetic Hydroxylapatite in Salt Solutions

The n d . . . h

final condition was approached from the supersaturated side and *the open circles* are those obtained for solubility in CO_2 containing salt solutions when the final condition was approached from the undersaturated side

obtained over the whole pH range in question which agreed with Bjerrum s values as well as could be expected if account is taken of the known formation of complexes the influence of different particle sizes and analysis errors Figure 42 has the same diagrammatic composition as in the other figures so along the ordinate you have the logarithm of the ratio between solubility found and that expected The points we shall look at first are the triangle signs which all fall in this area along the abscissa where the logarithm is zero that is where the relative solubility is one (1) Thus this is in agreement *with Bjerrum s formula This shows that the deviations* found in the saliva enamel experiments were not due to experimental errors

Equilibration Tests with Solutions Saturated with Calcium Carbonate

Of the salts other than calcium phosphate appearing in both dental enamel and saliva carbonates are predominant It seemed natural therefore to test the solubility of hydroxylapatite in the presence of the carbonate system This same figure shows the results of the equilibrium experiments *with the same hydroxylapatite in salt solutions which are simul*taneously saturated with calcium carbonate The points we find here roughly are along this same broken reference line which goes through the logarithmic diagram that is deviations of the same direction and the same order of magnitude as those found in the enamel and saliva experiments This was hydroxylapatite and calcium carbonate together in salt solutions

The comparability of the enamel apatite and the synthetic hydroxylapatite was checked by equilibrium experiments with the synthetic apatite in the saliva The same relative solubility was obtained as in the enamel and saliva experiments *Figure 43 shows the results with the points falling along* the reference line The same relative solubility was obtained as in the enamel and saliva experiments

Equilibration Test with Sodium Chloride Hydrochloric Acid Solutions

A number of tests with individual tooth enamels in *NaCl HCl* solutions showed again the same characteristics as corresponding tests with synthetic *hydroxylapatite mixed with 2 per cent calcium carbonate* I should perhaps try to elaborate somewhat on that though there were indeed very few experiments of that type There were some experiments with individual enamel samples in salt solutions well defined salt solutions free of carbonate and for comparison experiments with hydroxylapatite calcium carbonate mixtures of different calc[illegible] *[illegible]nate content If the same acid* solution was equilibrated with a ver[illegible] [illegible]plus of powdered enamel in [illegible] we got a higher final pH [illegible] surplus I think that would

mean that with the small surplus the calcium carbonate content was too small to increase the pH value and the relative solubility as much as with the greater surplus If these values for the enamel samples were compared with the corresponding values obtained with the same solutions and the same surplus amounts of hydroxylapatite calcium carbonate mixtures with 1 per cent calcium carbonate only then about the same pH levels and relative solubilities were obtained But if 5 per cent calcium carbonate were added instead to the synthetic hydroxylapatite then higher pH values and higher relative solubilities were obtained, and the marks fell higher up along the reference line

Dental enamel has according to many investigators been considered to

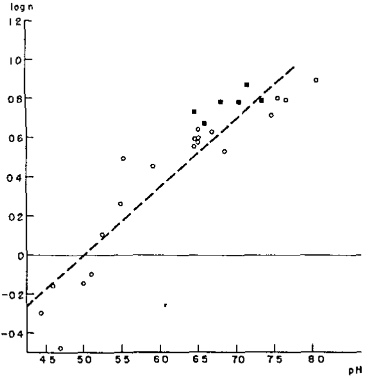


Fig 43 The Solubility of Synthetic Hydroxylapatite in Saliva

The ordinate gives the values for *log n* where *n* equals *the solubility found* divided by *the solubility expected from the formulae of Bjerrum*"[1] the abcissa the *pH* The *open circles* are the values obtained for saliva *plus* hydroxylapatite and *the solid squares* are those for saliva *plus* hydroxylapatite *plus* calcium carbonate

contain about 5 per cent calcium carbonate The experiments just mentioned here in addition to the x ray crystallographic analyses of Tovborg Jensen and Moller[253] point however to either lower carbonate content or a special form of binding of the CO_3 component I cannot argue on that Tovborg Jensen's theory was that calcium carbonate was bound in an amorphous form in irregular spaces in the hydroxylapatite system

The Effect of the Carbonic Acid System on the Solubility of Apatite

It seemed to be clear that it was the simultaneous presence of the carbonic acid system which in some way was the cause of the changed solubility of the apatite in both saliva and carbonate saturated salt solutions There was therefore reason to expect similar solubility deviations in other biological fluids under physiological CO tension for example in blood serum

The apparently very strong supersaturation of the blood with both calcium phosphate and calcium carbonate has naturally given rise to a large number of investigations and attempts at interpretation Most investigators have considered the explanation to lie in un ionized compounds of calcium with protein and other substances but the comprehensive literature on the subject contains so many contradictions that no definite conclusions can be drawn

In the well known previous investigation of Sendroy and Hastings[254] analysis data from equilibrium tests with blood serum and calcium phosphate made possible a rough recalculation according to Bjerrum s formulae This gave solubilities at the various pH levels which strongly resembled those of the saliva Figure 44 is the same type of diagram as in the previous figures You have the same line of reference and the points here are partly the recalculations and partly some experiments with human heparin plasma and horse serum The field of points falls along the reference line although many of them are below There is also at the circle in Figure 44 the result of a calculation for the normal values of human plasma, that is the calcium inorganic phosphorus pH and ionic strength of normal human plasma This calculation was performed according to Bjerrum s formula for precipitation and the circle happens to fall just on the reference line These results must of course be judged with great care but they seem to form an interesting parallel to the solubility tests with saliva and car

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[253]Tovborg Jensen A and Moller A Investigations in the Properties of Tooth Enamel by Means of X Rays *Acta Odont Scandina* 6 7 18 (1944)

[254]Sendroy J Jr and Hastings, A B II Studies of the Solubility of Calcium Salts The Solubility of Tertiary Calcium Phosphate in Salt Solutions and Biological Fluids *J Biol Chem* 71 783 796 (1927)

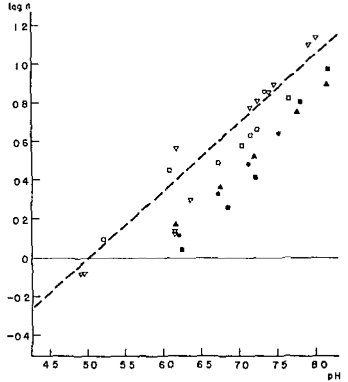


Fig 44 The Solubility of Hydroxylapatite in Blood Serum

[illegible] Iroy and Hastings [illegible] for horse serum *plus* calcium phosphate [illegible] excess phosphate *the open triangles* are the data (Ericsson) for horse serum *plus* hydroxylapatite *the open squares* are the data (Ericsson) for human plasma *plus* hydroxylapatite and *the open circles* are the data calculated (Ericsson) for the normal values of human serum

bonate saturated salt solutions and they seem to point to the assumption that the carbonic acid system common to all these liquids has a determining influence on the solubility of calcium phosphate

The mechanism of this carbonate effect on the calcium phosphate solubility is by no means clear but some facts seem to speak in favor of Greenwald's theory of un ionized calcium phospho-carbonate complexes Green

wald[155] *made some experiments* and calculations which led him to the assumption of a complex ($Ca_2 \bullet H \bullet PO_4 \bullet CO_3$) and after the dissociation of a hydrogen ion ($(Ca_2 \bullet PO_4 \bullet CO_3)^-$), Greenwald found also, an increased solubility of *calcium carbonate in the presence of the phosphoric* acid system as well as the increased solubility of calcium phosphate in the presence of the carbonic acid system

These were the points that I wanted to present, and now I would rather hear what you gentlemen have to say

Conference Discussion

Armstrong Thank you, Dr Ericsson Dr Hodge, would you like to speak now?

Hodge I presented quite a selection of Dr Ericsson's data last year, you may perhaps remember in plotting calcium phosphate pH relationships in solution His data were particularly pleasing to handle because they were some of the ones that fitted the lines best Apparently, they were very carefully and exactly done experiments, so that I have a high regard for the numbers that he has just been showing us here

As far as the Bjerrum s formulation goes, I don t see any great case to be made for it It looks to me like an attempt to describe the solubility relationships in a highly improbable manner If it is true, it calls for a reaction of about the ninth order, as I remember it

Ericsson In relation to Dr Hodge s remark I should perhaps, remind you of Bjerrum s finding that he could not arrive at the same concentration when he approached equilibrium from the supersaturated side as from the under saturated side There was always a gap between the concentration found in those two cases even if the equilibration was continued for months

Hodge I think the really important point, or at least an important point to see in these data is that we can find the same solubility behavior for powdered human enamel and for synthetic hydroxylapatite, is that right?

Ericsson Yes *but these investigations should* be continued of course They are few enough, but they are consistent thus far

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[155] Greenwald I The Effect of Phosphate on the Solubility of Calcium Carbonate and of Bicarbonate on the Solubility of Calcium and Magnesium Phosphates *J Biol Chem* **161** 697 704 (1945)

SOLUBILITY STUDIES WITH SYNTHETIC HYDROXYLAPATITE

WILLIAM F. NEUMAN

From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Neuman: I would like to make a few parenthetical remarks and present a couple of figures, if I may. Figure 45 doesn't add much to our understanding, but it seems that either the concept of solubility should be abandoned or proved. Mr. Levinskas and I, too, have performed a few solubility experiments.

Our first problem was extremely variable results. We were using synthetic apatite as the solid phase and found that if we centrifuged suspensions of apatite we could get different values at different depths of the solution. We couldn't get the solid down completely. Apparently there is a great deal of difficulty in removing suspended solid of microscopic size. These phosphate crystals powder during equilibration. We therefore switched to cellophane bags and then had trouble with bacterial infection which gave us very erroneous results.

Finally, with aseptic technic and cellophane bags, we got down to a group of points that I have tried to indicate in the graph. One trouble is that cellophane bags occasionally leak. The only way one can be sure is to do thirty experiments and have twenty of them that can be utilized.

Choucroun: Did you sterilize them?

Neuman: Yes, but I think it probably could have been done better.

Choucroun: You can autoclave cellophane bags, too.

Neuman: They get pretty rough treatment throughout the shaking period and subsequent handling. We did finally get values that are really quite low. In the graph they are expressed as moles per liter. These experiments were done in saline with synthetic apatite, using approximately the ionic strength of plasma. We have used the best activity figures obtainable for the activities of individual ions. Finally, we used the combined total activities of the several phosphate ions as a convenient way of expressing the results, but because the relationships at the moment are not clear, we are presenting the data in molar quantities.

Hastings: What is the pH of this?

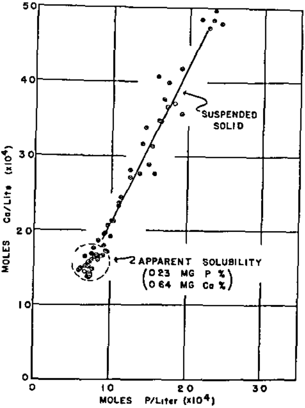


Fig 45 An Attempt to Determine the Solubility of Hydroxylapatite

Most of the values located within the broken circle were obtained using dialysis bags All of the other values involved analysis of supernates following slow speed centrifugation

Neuman The pH values were between 6 85 and 6 89, if I remember correctly

The next graph (Figure 46) shows two effects varying the solid and varying the Ca/P ratio It is interesting to note that with increasing solid phase, the calcium concentration increased and the phosphate concentration did not Apparently, any ion product that we could calculate here would be a function of the amount of solid phase present

Then, using one gram per liter, which were the data given in the previous graph calcium was added in varying amounts, and phosphate was added in varying amounts The results nicely intersect the previous curve, where neither ion was added The addition of calcium always causes a decrease

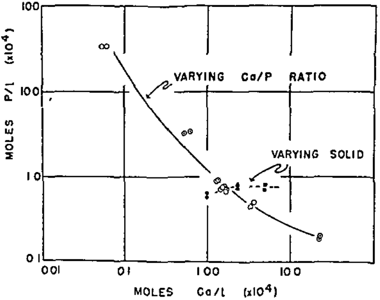


Fig 46 The Effect of Added Calcium or Phosphate on the Solubility of Hydroxylapatite

Also included are data obtained with varying amounts of solid phase *The solid circle the open circle with a dot in the center the solid triangle* and *the solid square* represent 0 3 1 3 and 10 grams/liter respectively

in the final pH and the addition of phosphate to the solution although we start at neutrality causes the pH to go up

This begins to look like some kind of solubility relationship that would be consistent with your set of data Dr Ericsson I just wondered if you had tried any experiments using varying amounts of solid phase The reason I make this point is that the curves that Dr Hodge showed a couple of years ago were the reverse of anything one might predict from solubility When the calcium went up the phosphate went up and when the calcium went down the phosphate went down In this single instance here, the *high calcium is associated with a low phosphate and the high phosphate* is associated with a low calcium This gives some reason to hope for future interpretations It is quite obvious that if we now changed the amount of solid and repeat the calcium phosphate variation we are just going to have another curve moved up the axis a way As yet we haven t been brave enough to add CO_2 to this system

Conference Discussion

Neuman Dr Ericsson have you done experiments with varying amounts of solid?

Ericsson Yes I have although not with very greatly varying amounts of solid and they gave consistent results There is a question as to the preparation of hydroxylapatite How do you prepare it?

Neuman This is a commercial preparation on which we have x ray data and electron photomicrographs The Ca/P ratio of this material is 2 15

Ericsson There is no carbonate in it then?

Neuman It is as nearly CO_2 free as we can get it

Armstrong I would like to ask about the commercial source of hydroxylapatite Who makes hydroxylapatite commercially?

Hodge It is labeled 'tricalcium phosphate' (Laughter)

Hendricks We knew that years ago

Neuman That is one of the problems Bone is so variable We wanted to have a source of material that is constant We, therefore purchased 10 pounds of hydroxylapatite Now all our experiments are directly comparable We are going through the 10 pounds of hydroxylapatite

Ericsson In fact I made some recalculations after Holt La Mer and Chown s [56] results which were obtained with commercial tricalcium phosphate in well defined CO free salt solutions, that is I made the calculations according to Bjerrum s formulae and found excellent agreement with his solubility figures for hydroxylapatite

Hastings Dr Ericsson one of the other things that we encountered was that we could get very good solubility products with calcite in salt solutions at different ionic strength varying pH and so forth A little phosphate would interfere with the precipitation from the high side, I mean from the supersaturated side

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a Holt L E Jr La Mer V K and Chown H B Studies in Calcification I The Solubility Product of Secondary and Tertiary Calcium Phosphate Under Various Conditions *J Biol Chem* 64 509 565 (1925)

b Holt L E Jr La Mer V K and Chown, H B Studies in Calcification II Delayed Equilibrium Between the Calcium Phosphates and Its Biological Significance *J Biol Chem* 64 567 578 (1925)

c Holt L E Jr Studies in Calcification III A Quantitative Study of the Equilibria Concerned With the *Calcification of Bone* *J Biol Chem* 64 579 587 (1925)

Ericsson I never made any experiments with calcite

Hastings There is one other minor point In recalculating those data of ours, did you allow for our better knowledge of what the calcium ion in the serum is, or did you use the plasma calcium data directly

Ericsson No You see, I proceeded from the certainly wrong assumption that all analyzed calcium and inorganic phosphorus were ionized and hoped to get such deviations that they might give a clue to the reason

Hastings Yes That was the reason we started doing these experiments in the first place, too,—using the solubility of difficultly soluble salts in order to get the ion product in the plasma—only to find the result which you just quoted

THE LOCATION AND THE TURNOVER OF THE SODIUM OF BONE[257]

I S EDELMAN, A H JAMES, and F D MOORE

From the Department of Surgery of the Harvard Medical School and from the Surgical Service of the Peter Bent Brigham Hospital Boston Massachusetts

Armstrong I should now like to hear from Dr Edelman who has some information as to the location and the turnover of the sodium of bone

Edelman Actually my getting involved with bone was inadvertent Our laboratory has been interested in the distribution of water and electrolytes in the intact organism using tracer methods [258 59 260]

One of the difficulties in calculating the distribution of ions from isotope dilution is the fact that the classical concept of dividing an organism into intracellular interstitial and plasma fluid compartments is erroneous One of the reasons that this simple three compartment system is invalid is that bone made up mostly of extracellular solids is not freely permeable to all suitable tracers

The Content of Sodium in Human Bone

From previous analyses [61] we know that there is a considerable amount of sodium and water in bone For a 70 kg human we expected that about 1000 meq of sodium (approximately 30 per cent of body sodium) and two to five liters of water (approximately 8 per cent of body water) would be in bone The following questions therefore remain to be answered how much of bone sodium and water is part of the metabolic pool? How much can contribute to the formation of edema? How much could be used to re

[57]This work was generously supported by a grant from the Massachusetts Heart Association

[58]Moore F D Determination of Total Body Water and Solids with Isotopes *Science* 104 157 160 (1946)

[59]Schloerb P R Friis Hansen B J Edelman I S Solomon A K and Moore F D The Measurement of Total Body Water in the Human Subject by Deuterium Oxide Dilution *J Clin Investigation* 29 1296 1310 (1950)

[60]Corsa L Jr Olney J M Jr Steenburg R W Ball M R and Moore F D The Measurement of Exchangeable Potassium in Man by Isotope Dilution *J Clin Investigation* 29 1280 1295 (1950)

[61]Shohl A T *Mineral Metabolism* American Chemical Society Monograph Series Reinhold Publishing Corp New York (1939)

place extracellular water and sodium in acute negative sodium balance and/or acute negative water balance?

The first difficulty we faced was that there are relatively few analyses on human bone available in the literature Bergstrom[6] at Children's Hospital in Boston has data indicating that the figures for bone sodium have to be revised upwards considerably and that the reason for the low figures in the literature is that sodium is co precipitated with calcium when oxalate is added to bone extracts Our own analyses corroborate those obtained by Bergstrom In any case we now estimate that there is between 1500 to 2000 meq of sodium in bone in the human

The Exchangeable Sodium of Human Bone

The next problem was to determine how much of this sodium is exchangeable since we would predict that the non exchangeable fraction would not be available for metabolic displacement The plan of the experiments was to use the ratio of the specific activity of bone sodium to serum sodium as the index of exchange after distribution equilibrium was attained That distribution equilibrium is achieved by 24 hours post injection is evident from the data in Table XIV The ratio of tissue specific activities to serum sodium specific activity is approximately 1 0 in all tissues except bone by 24 hours post injection of Na^{24} In other words tissue sodium for the most part is freely exchangeable with serum sodium These data corroborate similar observations reported previously [63] However from Table XV it is evident that complete exchange of sodium in dog bone does not take place in 24 hours The maximum exchange recorded is about 50 per cent We felt that two interpretations of these data could be made On the one hand 50 per cent of bone sodium may be truly non exchangeable and we have reached the limit of the amount that will exchange or on the other, we might be dealing with slow but continued exchange which would result in complete equilibration with the tracer if the experiment were continued for a long enough period of time

Follis When you talk of bone you are talking of bone plus marrow?

Edelman Of those listed in Table XV the femur was marrow free the rib was analyzed as cortex plus marrow and the skull sample consisted of the external plate

Now then in order to study sodium exchange over long time intervals

[6] Bergstrom W Personal communication

[63] Manery J F and Bale W F The Penetration of Radioactive Sodium and Phosphorus into Extra and Intracellular Phases of Tissues *Am J Physiol* **132** 215 231 (1941)

TABLE XIV

Tissue Uptake of Na^{24} in Dogs and Humans*

Subject	Tissue	Tissue S A / Serum S A
Dog #37	Skin	1 00
L S	Skin	0 94
Dog #37	Skeletal Muscle	1 04
L S	Skeletal Muscle	0 98
J W	Smooth Muscle (Gastric)	1 00
Dog #54	Cardiac Muscle	1 15
Dog #49	Cardiac Muscle	0 99
Dog #54	Liver	1 01
Dog #54	Kidney	1 21
Dog #49	Brain	0 99
J W	Gastric Mucosa	1 07
R K	Erythrocytes	1 02
Dog #28	Erythrocytes	0 94

Equilibration time = 24 hours

TABLE XV

Penetration of Na^{24} into Bone in Dogs*

Subject	Source	Tissue S A / Serum S A
#36	Rib	0 52
#37	Rib	0 53
#49	Skull	0 36
#49	Femur	0 52

*Equilibration time = 24 hours

we had to use sodium[22], which has a three year half life as compared with a 15 hour half life for sodium[24] In addition it was essential that the serum specific activity be relatively constant in that a rapidly falling serum activity could introduce an artefact in the tissue serum ratios This condition was satisfactorily met by maintaining the dogs on a rice salt poor milk and sugar diet The animals lost little weight and were in good health by inspection Sodium[22] was injected intravenously into two dogs The serum specific activity decreased only 12 per cent at the end of a month This meant that the bone was exposed to a constant tracer environment

The results are summarized in Table XVI The fraction of bone sodium exchanged did not increase with time About 50 percent of the sodium exchanges in 24 hours and the same ratio was obtained at the end of a month The depression in specific activity ratio in dog 78 at 24 days is probably partly experimental error and not significant I would guess that if one carried the experiment to the point where all of the original bone has been replaced by new bone, you eventually would get a ratio of 1 0 But certainly for any interval measured in months there is no deviation from the exchange of 50 per cent

Armstrong Did you biopsy the skeleton from time to time? You show several observations on the skeleton from one to 30 days

Edelman Yes We operated on these animals under aseptic conditions and the dogs did not receive any saline or plasma during the period of

TABLE XVI

Penetration of Na[22] into Bone in Dogs *

Dog #28			Dog #78		
Equilibration time	Source	Tissue S A / Serum S A	Equilibration time	Source	Tissue S A / Serum S A
Days			*Days*		
1	Rib	0 27	1	Rib	0 46
3	Rib	0 49	8	Rib	0 44
8	Rib	0 45	17	Rib	0 48
15	Rib	0 49	24	Rib	0 37
29	Rib	0 49	24	Skull	0 35
29	Skull	0 43	30	Rib	0 43

*Maintained on low sodium (rice diet) for period of study

study They withstood the operations nicely We simply obtained a sample of bone and a sample of blood at the times indicated in Table XVI and they were analyzed simultaneously Skull samples were not taken earlier in the experiment in that we wanted to minimize surgical trauma and to avoid losing the animals in the first weeks of study

The Distribution of Sodium in Bone

We now have the question of how to interpret these data from the viewpoint of bone structure By chemical analyses of both dog and human bone we found about 220 meq of sodium per kilogram of fat free dry bone which is a large amount As a first approximation as to how these fractions (exchangeable and non exchangeable) might be distributed we made the assumption that Dr Hastings has used for other tissues, namely, that all of the chloride is extracellular and that all of the sodium accounted for by chloride would be freely exchangeable From chemical analyses we found that the ratio of sodium in association with chloride to total bone sodium was only 0 10 to 0 20, in other words, free extracellular sodium (based on chloride) does not account for all of the exchangeable sodium so that regardless of marrow content there is a considerable amount of sodium which must be part of the matrix solids which is exchangeable

It may be as Dr Hendricks suggests that the sodium is on the surface of the apatite crystal lattice or at least a fraction of it is, and perhaps there is another fraction in association with other molecules On Dr Hastings suggestion we calculated that there are about 188 meq of citrate per kilogram of dry bone based on the figures in the literature Thus there is more than enough citrate to account for all the exchangeable sodium

In addition we calculated the molar ratio of exchangeable calcium to excess exchangeable sodium based on some of the published figures for maximum calcium exchange, which seems to be about 25 per cent of the bone calcium Is that right, Dr Hodge?

Hodge That is the maximum, yes

Edelman Even with that maximal figure the molar ratio of exchangeable calcium to excess exchangeable sodium is 20 The molar ratio of total calcium to non chloride sodium (excess sodium) is about 30, which suggests that sodium occupies more of the lattice surface than calcium

Hastings Say that again, please

Edelman By subtraction of the free extracellular sodium, based on association with chloride, from exchangeable fraction of sodium, we get the excess sodium of bone matrix which is exchangeable The molar ratio of exchangeable calcium to this excess exchangeable sodium is 20, while the

molar ratio of total calcium to total excess sodium is 30 In other words there is proportionately less exchangeable calcium as compared with sodium Similarly the molar ratio of exchangeable phosphate to exchangeable sodium using the figure of 20 per cent exchange for phosphate is 9 8

While these ratios do not define the distribution of sodium in bone they do indicate that we are dealing with a heterogeneous system in which only a given portion of sodium is available for exchange From the viewpoint of metabolic implications the problem that faces us is how much of the exchangeable sodium is available for displacement from bone into the free extracellular pool and if such shifts occur then we need to define the critical determinants of such shifts

Conference Discussion

Follis Will you tell a layman what is the concentration of sodium in terms of per cent in dry fat free bone?

Edelman In human and dog bone there are about 220 mill equivalents

Follis I don t understand milliequivalents in that sense

Edelman It is about 220 milliequivalents per kilogram of dry fat free bone I might also say that analytic results vary considerably depending upon the type of bone There are large variations of sodium and water content in different bones and it is only when you correct to dry weight that consistent figures are obtained

Neuman You divide by 4 or 5 tenths per cent for milliequivalents

Hastings Five hundredths I would like to hear Dr Hendricks comment on what that sodium might mean

Hendricks The sodium is and always has been one of the nice arguments for surface phenomena Of course in Logan and Taylor s [44] work they found that the sodium was immediately taken off the surface on resolution in lactic acid The fact that it is not all exchangeable is not necessarily confusing in that even if it is on the surface it is a question of whether you get at all the surface or do not I believe the *in vivo* experiments on calcium and phosphorus do not get as large a turnover as you would expect from the possible surface of the bone They are I believe of the order generally of half the figures that you were giving Dr Edelman namely more down to the order of 10 per cent of the calcium and 10 per cent of the phos

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[44] Logan M A and Taylor H L Solubility of Bone Salt III Partial Solution of Bone and Carbonate Containing Calcium Phosphate Precipitates *J Biol Chem* 125 391-397 (1938)

phate that is available in the turnover there The idea is that the sodium which is not readily turned over is buried down in some cracks of the same nature as the ones that were shown with carbonate This would be the interpretation there I don t believe that the other investigators who are particularly arguing about carbonate apatite would be averse to having sodium external on the surface of the lattice They would probably have no feeling one way or the other

McConnell I would agree to that 100 per cent unless somebody found it in something crystalline like francolite Furthermore although it has not come to light previously there is one other point on which Dr Hendricks and I would probably agree 100 per cent and since there have been so many points on which our opinions are quite divergent perhaps this one particular point should be emphasized because it does diverge from some of the work that is being currently done in Europe

I believe that no matter what the composition of carbonate apatite is—and we will admit that we don t know what the stoichiometry of it is for the moment—it certainly is not $Ca_{10}CO_3(PO_4)_6$ and all calculations which involve any such nonexistent compound as this are bound to be fallacious There can be no such compound and Dr Gruner and I demonstrated that many years ago [265] Crystochemically there can be no such compound

Bartter If you take the $CaCO_3$ and put it to one side is it all right?

McConnell No you are just juggling the formula and rewriting it $3Ca_3(PO_4)_2 \cdot CaCO_3$ There is absolutely no such compound and although Dr Hendricks and I have lots of differences of opinion we certainly concur on that

Hendricks This is the reason that one structure turns back into arguments of getting carbonate in place of phosphate and still leaving the water or hydroxyl or the various other things out in position, in other words that is the reason that the whole argument turned back on getting carbonate in place of the phosphate

There is one point though I would like to raise about these skeletal replacements I believe it was pointed out here last year that the reason that you use alkaline KOH instead of NaOH in Gabriel s system for removing protein from bone is that if you use NaOH it is markedly taken up by your sample It will go up to the order of a per cent by weight in the sample It is uniquely concerned with the fact that sodium and calcium are identically the same size although they differ in charge Each has a radius

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[265] Gruner J W and McConnell D The Problem of the Carbonate Apatites The Structure of Francolite, *Z Krist* 97A 208 215 (1937)

of 0.98 Angstrom units Potassium with a radius of 1.35 Angstrom units is too big to fit into the calcium hole and doesn't go on the surface in other words the surface does have one very exact geometry It excludes potassium and it accepts sodium

Edelman Are those the hydrated radii?

Hendricks No, just the radius from normal ions The question of hydrated ions is more difficult

McConnell We don't want to hear about that one

Armstrong Are there any further ideas to be expressed with regard to the exchange of sodium in the skeleton? I would like to suggest that at different times, an element may be exchangeable and nonexchangeable Certainly this is true with regard to calcium If you give radiocalcium to rats the radio isotope will become situated in the skeleton undoubtedly by exchange for calcium[40] ions With the passage of time the specific activity of the bone reaches a limiting low value from which it does not further decline This finding shows that the calcium ions which enter the skeleton become less freely exchangeable after a time

Hendricks It is the phenomenon that Dr Dallemagne calls recrystallization

STUDIES ON RADIOPHOSPHATE EXCHANGE IN BONES OF RATS

WILLIAM F NEUMAN

From the Division of Pharmacology and Toxicology Department of Radiation Biology School of Medicine and Dentistry University of Rochester Rochester New York

Neuman I am getting embarrassed but I have another figure to show

Armstrong We still have a few minutes I am going to ask Dr Neuman to present his data after which I am going to call on Dr Hodge

Neuman This is just an experiment in which we injected rats with radiophosphate took out the bones at various time intervals and placed them in an inert buffer to see how much of the radiophosphate will back exchange In Figure 47 you can see with the passage of time in the animals the phosphate becomes less and less desorbable I have interpreted this as evidence for the occurrence of the recrystallization process *in vivo* in the animal

Conference Discussion

Armstrong How rapidly does this process occur?

Neuman These are fairly young animals (about 100 grams in body weight) The experimental period covered 18 days

Armstrong It would be a little less than 10 days

Copp I should think the situation would be a little different with growing animals where new crystals of bone salt are being laid down

Neuman Yes Well we are trying this with animals of different ages

Copp We found that was true with calcium The radiocalcium deposited in the skeleton of young animals was not removed to any appreciable extent whereas in adult animals there was considerable loss in the early days after administration

Neuman That would be expected on the basis of recrystallization With every recrystallization the crystal becomes more stable Therefore the rate of recrystallization would fall with age But I don t think this concept of recrystallization is adequate to explain all the physiological data

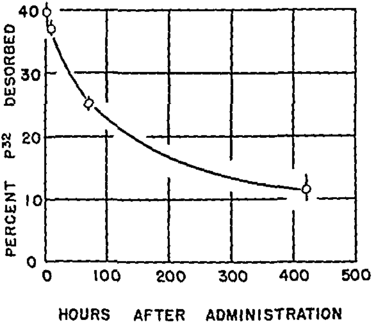


Fig 47 The Decrease in Desorbable Radiophosphate of Powdered Femur with Increasing Time After Administration of Isotope

Copp I don't think it is necessary to have recrystallization, is it, Dr Hendricks? In the young animal and in areas of new bone growth there is *actual* formation of new crystals of bone salt. Another point we observed with respect to exchangeable calcium in adult animals was that the turnover was considerably less than 1 per cent *in vivo*, indicating that a good deal of the bone calcium is not available for ready exchange with the extracellular calcium

Kochakian All this seems to take place in this crystal structure. Does the protein have anything to do with this?

Hendricks I would say that protein doesn't enter into it or do anything except to interfere with the extent to which you can get at the surface. I would sort of suspect that it interferes markedly in a particular bone as to whether the ion can get in there to the surface and produce the exchange

Armstrong In some situations protein matrix possibly accelerates exchange of the ions of the mineral phase because a more ready access of the body fluids is permitted. This situation certainly is seen in the rachitic bone epiphysis

THE SIGNIFICANCE OF THE SKELETAL DEPOSITION OF FLUORIDE[266]

HAROLD C HODGE

From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry University of Rochester Rochester New York

Armstrong I think Dr Hodge has some information about the turnover of fluoride in the bone

Hodge I have been trying to persuade Dr Armstrong to go ahead and give his data I think they are closer to the topic we have been discussing This is a side issue

The point I want to talk about is our concept of the deposition of fluorides or what the skeleton does with the fluorides The reason this is timely is that there is a discussion in various parts of the country about the safety of fluoridation of water Since in long continued, high level fluoride exposures, people develop crippling fluorosis, preceded or accompanied by osteosclerosis the question about the health of the skeleton arises

The widespread use of fluorides in the drinking water of communities attempting to reduce the incidence of dental caries has prompted a steady reiteration of questions concerning the safety of this use Since one of the principal toxic effects observed when large amounts of fluorides are given for long periods is osteosclerosis and since bony changes are prominent in crippling fluorosis it is natural to inquire what the role of the skeleton may be *in the metabolism of fluorides At the moment our attention is directed* to problems of mineral metabolism rather than to problems of safety, and the questions regarding the hazards of water fluoridation will be mentioned only in passing

The Fate of Fluoride in the Human

Although much additional work needs to be done to clarify some of the important aspects of fluoride metabolism, it is possible at present to sketch in the broad outline of how the body handles fluorides (Figure 48) Fluoride taken into the body by any route and absorbed into the body fluids is

[266] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project Rochester New York

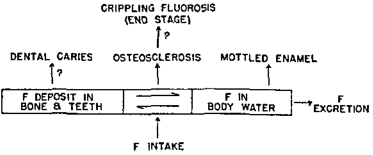


Fig 48 Diagrammatic Description of the Distribution of Absorbed Fluorides with Indications as to Some Effects

promptly disposed of almost in its entirety either by deposition in the skeleton or by excretion in the urine The promptness of this disposal should be stressed, in two or three hours the process is practically complete The relative magnitudes are also important subject to qualification the proportion in bone and urine is nearly enough 50 to 50 to justify remembering The fluoride concentration in the blood in the body fluids in muscle and in most of the soft tissue is very low—a few micrograms per hundred grams The fluoride ion concentration in the body fluids is important because this is probably a controlling factor in the hypoplastic changes exhibited by the ameloblasts associated with the fault in enamel formation described as mottled enamel Presumably the fluoride ion concentration is not the controlling factor in the reduction of dental caries the fluoride deposited in the tooth mineral is commonly believed to be the critical factor In the development of osteosclerosis, both fluoride ion and fluoride deposited in bone mineral may be important the fluoride ion may influence the production of osteoid or its properties or the intricate changes occurring during calcification and in addition the mineral crystals containing fluoride may differ sufficiently in physical chemical properties from hydroxylapatite crystals to be responsible for part of the osteosclerosis picture How these effects may interplay in the etiology of crippling fluorosis is not known

The Skeletal Deposition of Fluoride

The skeletal deposition of fluoride may be regarded as a sort of detoxification mechanism Presumably if substantial amounts of fluoride were not deposited in the skeleton higher concentrations of fluoride ion would be maintained for longer periods of time Furthermore there is no evidence that fluoride deposition *per se* is harmful The relationship between the

fluoride content of the bone and the fluoride concentration in the blood when fluoride intake is very low is not known

Skeletal deposition of fluoride occurs at all levels of fluoride administration There is a widely quoted opinion that the body tends to come into a sort of equilibrium state when exposed constantly to low doses of fluoride Thus McClure said There was no significant retention of fluorine in the bodies of these young adult men when total daily fluorine ingested did not exceed 4 0 5 0 mg daily [67] When sufficiently long periods of time are used in the evaluation of the retention of fluoride, the evidence appears to support overwhelmingly the concept of continued storage Machle and Largent [68] show a linear relationship between fluoride absorption and bone storage in a human taking relatively large doses (up to 20 milligrams daily) In this work nearly 50 per cent of the fluoride absorbed was deposited in bone Without attempting to review in detail the fairly extensive literature on fluoride retention the increase in the fluoride concentration with age in the skeletons of residents of Rochester N Y may be shown (Figure 49) I take pleasure in attributing the analytical data to Dr Frank Smith and Mr Dwight Gardner

Although there was a marked variation between individuals of the same chronological age there is a striking approximation to a linear relationship when the average bone fluoride concentrations for ten year age groups are plotted against the logarithm of the age This graph indicates a steady accumulation of skeletal fluoride throughout life even on relatively low levels of fluoride ingestion Rochester water contains about 0 06 p p m of fluoride and the daily intake of residents in food and water probably ranges from 0 5 to 1 0 mg About 90 cases were examined in the Rochester study[269], these together with the 23 analyses of Glock Lowater and Murray [270] constitute most of the available evidence on this point More data are needed

The Rapid Process of Fluoride Deposition in the Skeleton

It is probable that a rapid and a slow process are involved in fluoride de

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[267] McClure F J Nondental Physiological Effects of Trace Quantities of Fluorine in *Dental Caries and Fluorine* published by Am A Adv Sc Washington D C. (1946) pp 74-92

[268] Machle W and Largent E J The Absorption and Excretion of Fluoride II The Metabolism at High Levels of Intake *J Indust Hyg & Toxicol* 25 112 123 (1943)

[269] Smith F A Gardner D E and Hodge H C Skeletal Storage of Fluoride in the Human *A E C Quarterly Technical Report UR 200* pp 12 14 (Feb 11 1952) University of Rochester Rochester N Y 1952

[270] Glock G E Lowater F and Murray M M The Retention and Elimination of Fluorine in Bones *Biochem J* 35 1235 1239 (1941)

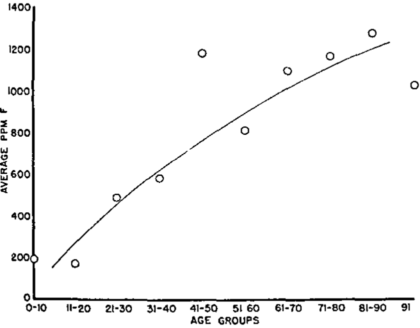


Fig. 49. The Increase in the Fluoride Content of Human Skeletal Samples with Age.

The data are those of Smith, Gardner and Hodge. The bone samples were taken at autopsy of residents of Rochester, N.Y. (drinking water contains 0.06 p.p.m. of fluoride).

position. The rapid process is presumably an exchange reaction in which the fluoride ions of the body fluid exchange with hydroxyl or bicarbonate ions of the surface of the mineral crystals. The speed of this reaction is well known; commercial filters are available that remove most of the fluoride when excessive amounts are present in water merely by allowing the water to flow over a bed of powdered bone. The surfaces of the crystals present large areas on which this reaction can occur. Neuman and colleagues[271] and Megirian *et al.*[272] have shown that fluoride competes with hydroxyl for positions on the mineral surfaces. Since the total amount of

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[271] Neuman, W. F., Neuman, M. W., Main, E. R., O'Leary, J., and Smith, F. A.: The Surface Chemistry of Bone. II. Fluoride Deposition. *J. Biol. Chem.* 187: 655, 661 (1950).

[272] Megirian, D., and Hodge, H. C.: Fluoride Exchange with Hydroxyl as the Mechanism of Fixation of Fluoride by Bone. *J. Dent. Res.* 30: 467 (1951).

surface will be more or less related to the total amount of mineral matter present this concept is in agreement with the finding of Savchuck and Armstrong [273] that the principal factor determining deposition and fixation of fluoride in a bone is its mineral content'

The Slow Process of Fluoride Deposition in the Skeleton

The slow process may plausibly be ascribed to the incorporation of fluoride ions into the crystal lattice At least three modes may be suggested a) growth b) recrystallization[274] and c) resorption and replacement of bone by osteoclastic and osteoblastic activity To the slow process may be attributed the gradual storage of fluoride even when very small quantities are taken in daily over a period of years

Choucroun That is dangerous

Hodge No it is not necessarily dangerous That is what I just finished saying there is no indication that the deposition of fluoride *per se* is a hazard

Now what if a person drank water containing one part per million of fluoride and stored all of it? He would if he lived to be 70 years old have in his skeleton almost pure fluorapatite

McConnell Francolite I beg your pardon (Laughter)

Hendricks He would be a geological specimen

Armstrong He would be a fossil (Laughter)

Neuman Does my memory serve me right? There was a cow on a very high fluoride intake and on *post mortem* analysis the bones proved to be almost pure fluorapatite?

Hodge There are a few samples reported in the literature with fluoride percentages that were upwards of 3 5 which would be theoretical for fluorapatite Anyway we have a logarithmic function here

The Removal of Fluoride from the Skeleton

The removal of fluoride from the skeleton also is characterized by two phases a rapid process and a slow process The rapid process most recently studied by Savchuck and Armstrong[273] probably results from an ex

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[273] Savchuck W B and Armstrong W D Metabolic Turnover of Fluoride by the Skeleton of the Rat *J Biol Chem* 193 575 585 (1951)

[274] Neuman W F and Mulryan B J The Surface Chemistry of Bone I Recrystallization *J Biol Chem* 185 705 712 (1950)

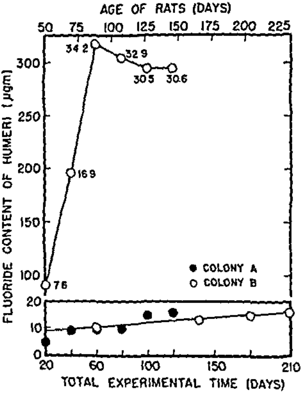


Fig 50 Tl e I l or de Co te t of Rat Bo e

Tl e da a a e t o e of Sa hu k and A m g Wean g ma e Sp ague Dawl a we e g en 20 p p m f flu d h d nk ng w e fo a 0-da pe d *upp ur)* The a o f e on o a on a ed abou 0 6 p p m of fluo d *l v ur* The numbe a ong he uppe u e a e e ma of the to a fluo e nake n m l g a n T e eep y u g to e boe o e upper cu e cc e pond t fl d r ons f abou 50 pe ce Ra sa d 20 40 and 60 da s af mo l f om e fl expo u e ho ed l dec a ng bore flu de alu [R pr d ed by perm n f m S u k W B an l A m ong W D Metab lu o er f F u de b e Skel n of e Ra *J B h n* 193 75 85 1951]

cha ge f hydrox l or l carbonate on n h flu ride ns of tl e cr stal u face (I g re 0) One n l at on f l l e ome on tl e requ ren ent f r per od f not e nt ou a ln trat o of fluor les n tl e rol f dental ca es (pro ded tl e p ence of fl or de on n tl e em el s es ntial) Tl e rap d process is demo trat l grapl all l p ott n tl e per cent ge of t re l fl or le on a logar tl n cale aga t the t ne n eek In the fr t n nth af r a period f fl r le exp ure the ur ar excret n s lerall l l er tl an tl e t t tren l tha l e l tl m l emat cal

precision over a period of two years (data of Largent and Heyroth[275]) The slow process as revealed by the straight line just described may be characterized by usual means, the half time constant is of the order of two years It is reasonable to think that the removal of fluorides in the slow process is accomplished by releasing to the body fluids lattice bound fluoride when recrystallization or osteoclastic activity destroys a mineral crystal

Summary

The skeletal deposition of fluorides may be regarded as a detoxification mechanism, bone-bound fluoride is not a hazard *per se* Fluoride is deposited in bone at all levels of fluoride intake and throughout the life of the individual Fluoride deposition and removal from the skeleton involve at least two processes the rapid phase, presumably an exchange reaction with hydroxyl or bicarbonate of the crystal surface, accounts for the initial changes, the slow phase a result of recrystallization, growth or cellular activity, is characterized by a half time constant in man of about two years

Conference Discussion

Hodge You notice, this is a logarithmic process, too If you will pardon me, I will make a pun When we think of fluoride and the skeleton we should remember that the log giveth and the log taketh away (Laughter)

Dr Neuman's data[271, 276] presented here in 1950, show that there is a very precise relationship between fluoride and hydroxyl ion concentration Mr David Megirian has confirmed Dr Neuman's data, the hydroxyl ion and the fluoride ion, as far as content in bone is concerned, are competitors for surface positions Dr Neuman has also shown that the bicarbonate competes directly with fluoride for a position on the surface of the bone crystals We have a fairly reasonable picture of what goes on in terms of the deposition, either by exchange, or by building into the lattice The point should be stressed that there is no evidence that fluoride deposited in the skeleton is hazardous, *per se*

Armstrong Dr Hodge, have you seen the latest number of the *Journal of Biological Chemistry*? The conclusions of a paper in the December 1951 number are quite contrary to some of those which you have reached[273]

Hodge I am surprised to hear you say that because I found your data

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[275]Largent, E J, and Heyroth F F The Absorption and Excretion of Fluorides III Further Observations on Metabolism of Fluorides at High Levels of Intake *J Indust Hyg & Toxicol* 31 134-138 (1949)

[276]Neuman, W F Bone as a Problem in Surface Chemistry *Trans Macy Conference on Metabolic Interrelations* 2 32 72 (1950)

to be in agreement with our conclusion I won t try to present your data but one of the principal conclusions that Dr Armstrong[*] came to was that the deposition of fluoride in the skeleton was proportional to the total amount of mineral matter present Is that right

Armstrong That is not the point that I thought you stressed

Hodge All right This obviously relates to the total surface that is present which makes good sense On which point did you disagree

Armstrong Well perhaps I had better cite the case Dr Hodge has shown data obtained from metabolic balance studies carried out by Largent[*3] which indicate given sufficient time after withdrawal of fluoride from the diet that the total fluoride of the skeleton would be mobilized and excreted I realize the hazards involved in translation from the rat to the human but I should like to refer to experiments in which rats were fed a high fluoride intake and then the fluoride supplement was withdrawn The observations were that fluoride content of the skeleton decreased for a time after which time there was no evidence of any further elimination of fluoride from the skeleton I think this is in harmony with what has been shown here today by Dr Edelman for sodium It is also in agreement with results obtained with radiocalcium This finding is another piece of evidence for the incarceration of an ion in a situation in bones from which it is not freely mobilizable

If you could produce some effect upon the skeleton which would accelerate the total turnover of the mineral phase then the fluoride or any other ion in the skeleton could be brought into contact with the body fluids and might again have a chance to be mobilized and excreted

Hendricks Is the question here one of time scale though Isn t the rat s time scale small compared with the two years—because it is the absolute time scale and not the biological time scale that is important isn t it?

Armstrong Yes However we could see that over a period of approximately 100 days there was no change in the fluoride content of the skeleton The analyses were made directly on the bones of the animal so that the complications of analyses of excreta were avoided

Hodge The period of fluoride feeding (Figure 50) was not quite 100 days, was it?

Armstrong It was 20 days in one group 110 days in another group and 40 days in another group The point is that direct skeletal analyses for fluoride showed no significant decrease in amount even though the fluoride content was much higher than normal, in periods of 20 40 and 110 days after withdrawal of the fluoride supplement

precision over a period of two years (data of Largent and Heyroth[275]). The slow process as revealed by the straight line just described may be characterized by usual means: the half time constant is of the order of two years. It is reasonable to think that the removal of fluorides in the slow process is accomplished by releasing to the body fluids lattice bound fluoride when recrystallization or osteoclastic activity destroys a mineral crystal.

Summary

The skeletal deposition of fluorides may be regarded as a detoxification mechanism; bone bound fluoride is not a hazard *per se*. Fluoride is deposited in bone at all levels of fluoride intake and throughout the life of the individual. Fluoride deposition and removal from the skeleton involve at least two processes: the rapid phase, presumably an exchange reaction with hydroxyl or bicarbonate of the crystal surface, accounts for the initial changes; the slow phase, a result of recrystallization, growth or cellular activity, is characterized by a half time constant in man of about two years.

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[275]Largent, E. J., and Heyroth, F. F.: The Absorption and Excretion of Fluorides. III. Further Observations on Metabolism of Fluorides at High Levels of Intake. *J. Indust. Hyg. & Toxicol.* 31: 134-138 (1949).

[276]Neuman, W. F.: Bone as a Problem in Surface Chemistry. *Trans. Macy Conference on Metabolic Interrelations* 2: 32-72 (1950).

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Armstrong It was 20 days in one group 110 days in another group and 40 days in another group The point is that direct skeletal analyses for fluoride showed no significant decrease in amount even though the fluoride content was much higher than normal in periods of 20 40 and 110 days after withdrawal of the fluoride supplement

Johnson I would like to ask what Dr Hodge means by "harm *per se*", in contrast to what else? There is something that I have missed very much in this conference namely the realization that there is a *difference in* matrices, the protein component with which all of this mineral physiology and chemistry is associated All osteoids or matrices are not alike, and in *the one case of fluoride poisoning that I have seen*, there is a very much disturbed matrix production That is the reason I want to know why the statement 'no harm *per se*' I think that fluoride contained in skeleton or present in the skeleton in large amounts during the period when the matrix is being synthesized may well be responsible for a highly abnormal matrix and result in a highly abnormal skeleton

Follis Abnormal in what sense?

Johnson Structurally

Follis Well, I mean how structurally?

Johnson The gross observation of the massive increase in weight and specific gravity which is expressed as osteosclerosis, the microscopic picture of abnormal Haversian patterns failure of the Haversian systems to adhere together, laying down of a matrix material that you would call osteoid but does not have the normal visual texture, being granular and lumpy The reticulum collagen and polysaccharide components do not stain clearly, as in normal bone At times it looks as though the two proteins, collagen and homogenous polysaccharide protein, are independently desposited without *being welded into a single new substance osteoid Sometimes the* involved trabeculae look parboiled in disorganization We have ignored all the way through this discussion, the fact that all osteoid is not alike I think *that is going to come up for discussion some day*

Armstrong I do not mean to express any disagreement about the main *conclusion reached by Dr Hodge as to the—well, shall I say* innocuousness of skeletally deposited fluoride The notion that the skeleton is a mechanism for detoxification is a perfectly valid one The skeleton has a more primitive function, perhaps *a more biologically important function*, than that of giving rigidity to the body It not only is able to remove dangerous substances from the circulation, but it can also contribute ions to the vital fluids or to the environment of the more vital processes in the body as well

Shorr Did these rats continue to grow well, Dr Armstrong?

Armstrong There is much information as to how much fluoride is needed to prevent animals from doing well We used lesser amounts than the minimum toxic level *The animals* were in good condition as regards body weight and skeletal growth

Gutman Perhaps some of you have perused Roholm's monograph on fluorosis He was a Danish investigator who was particularly interested in the results of fluorosis in cryolite workers in Denmark and Greenland Some of the effects of chronic fluoride intoxication to be sure at much higher levels of intake than you are dealing with are certainly impressive X rays, for example show very extensive—let's call it calcification for the moment—of tendons and muscle sheaths extensive arthritis of the spine with rigidity of the entire spine and marked osteogenesis The final picture of fluorosis may resemble advanced Paget's disease The fluoride content of those bones was exceedingly high I would not say that fluoride was wholly innocuous in the skeleton under such circumstances

Armstrong There is no doubt that high intakes of fluoride can produce deleterious effects I am persuaded from every bit of evidence I can gather (and I am sure that Dr Hodge and all the other persons who have looked into the subject would be in agreement) that there is only one possible ill effect that can result from the ingestion of water containing any reasonable concentration of fluoride That condition is mottled enamel The most convincing evidence that one can bring to bear on this subject is the fact that there are, perhaps a million people living in the United States who have for many years been using a water with a fluoride content considerably higher than that which is the recommended amount to be added to water So far as I know no confirmed case of skeletal fluorosis has been described in this country

Johnson There is at least one that I know of He was autopsied in the Army during the war and has been mentioned in the literature by several people,[277 278] and it is that one on which I saw the material and on which I based my comments about abnormal osteoid matrix It was a case[279] from Texas which turned up as an incidental finding in a uremic boy raised in an area notorious for fluorine mottling of teeth (which this boy also had due to as much as 12 parts per million of fluorine in the water) And by the way, the uremic disturbance in bone was distinct from and not to be confused with the fluorine lesions

Armstrong Do you know the fluoride content of the bone specimens?

Johnson The analysis of the bones in this case showed specific gravity,

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[277] Coley Bradley L *Neoplasms of Bone and Related Conditions* Paul B Hoeber New York p [illegible] (1949)

[278] Linsman J F and McMurray C A Fluoride Osteosclerosis from Drinking Water *Radiology* 40 474–484 (1943)

[279] Wolff W A and Kerr E G The Composition of Human Bone in Chronic Fluoride Poisoning *Am J M Sc* 195 493–497 (1938)

2 196 (normal control was 1 838) sternum 0 69 per cent fluorine in fat free bone ash vertebral 0 75 per cent and teeth 0 45 per cent The bone contains about three times as much fluorine as is reported for upper limits of normal (normal range 0 02 per cent to 0 25 per cent)

Howard How do the cryolite workers get in their fluorine? Is it through their lungs?

Gutman Cryolite is an aluminum fluoride

Howard But how does it get in?

Gutman By inhalation Cryolite is used to give porcelain its glaze and it is ground in before baking Exposure is mostly by inhalation The same applies to the cryolite miners

Johnson If we grant that fluorine can interfere with the physiology of the enamel cells how do we decide that it cannot interfere with the same physiology in the osteoblast? That is what the whole discussion turns on You already have fluorine in your bone salt crystal and you say that there is a slow rate of exchange Then why is not this exchanging fluorine capable of influencing osteoblastic function in the immediate neighborhood? Where is the evidence for a difference between the amount that is needed to affect the ameloblasts and the amount needed to affect the osteoblasts?

COMPOSITION OF TEETH IN RELATION TO CARIES SUSCEPTIBILITY[290]

ALBERT E SOBEL

From the Department of Biochemistry The Jewish Hospital of Brooklyn, Brooklyn N Y

Armstrong Dr Sobel has a few remarks

Sobel I would just like to point out that there might be another approach to the caries problem than fluorides, which probably does the same thing to teeth as fluorides This approach stems back to observations by Logan and Taylor,[291] Dallemagne *et al*,[292 293] Hendricks and Hill,[294] and Hardwick[295] that carbonate is preferentially dissolved from phosphate in bone and tooth minerals The assumption therefore was made that a high carbonate tooth would be more caries susceptible than a low carbonate tooth

We proceeded to produce the two types of teeth by putting a group of cotton rats, 16 days old, on two types of diet one high in calcium producing high carbonate, and one low in calcium producing low carbonate teeth [56] We then superimposed after a preliminary period on this diet the caries producing diet of Shaw and his colleagues [297] As a matter of fact Professor James Shaw has done the caries index studies on these rats at the Harvard School of Dental Medicine You will note from Table XVII that

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[290]This work was supported by the National Institute of Dental Research of the National Institutes of Health U S Public Health Service Earlier phases of this study were supported by the Williams-Waterman Foundation

[291]Logan M A and Taylor H L Solubility of Bone Salt III Partial Solution of Bone and Carbonate Containing Calcium Phosphate Precipitates *J Biol Chem* 125 391 397 (1938)

[292]Dallemagne M J and Brasseur, H The Diffraction of X rays by the Mineral Substance of Bone *Bull Soc Roy Sci Liege* 11 451 and 488 (1942)

[293]Brasseur H, Dallemagne M J, and Melon, J Chemical Nature of Salts from Bones and Teeth of Tricalcium Phosphate Precipitates *Nature* 157 453 (1946)

[294]Hendricks S B and Hill W L The Nature of Bone and Phosphate Rock *Trans Macy Conference on Metabolic Interrelations* 3 173 189 (1951)

[295]Hardwick J L Enamel Caries A Chemico physical Hypothesis Phase One—Chemical *Brit Dent J* 87 137 142 (1949)

[56]Sobel A E The Local Factor in Calcification *Trans Macy Conference on Metabolic Interrelations* 2 113 143 (1950)

[297]Schweigert, B S, Potts E, Shaw J H Zepplin M and Phillips P H Dental Caries in the Cotton Rat VIII Further Studies on the Dietary Effects of Carbohydrate Protein and Fat on the Incidence and Extent of Carious Lesions *J Nutrition* 32 405 412 (1946)

TABLE XVII

Influence of Diet on Caries Susceptibility

Diet	Number of Animals	Number of Carious Lesions	Extent of Carious Lesions
High Ca, Low PO_4	41	10 8	22 5
Low Ca, High PO_4	27	2 7	5 6

the number of carious lesions is four times as great in the rats on the high calcium diet with the high carbonate teeth, than in the other group with low carbonate teeth, and at the same time, the extent of carious lesions is about four times as great

We used groups of 41 and 27 rats (Table XVII) They were split litters I suppose one ought to look at a larger series, but at least in view of the statement of Dr Hodge today, that carbonate and fluoride compete with each other, it is quite possible that fluoride can achieve the same composition change resulting in a low carbonate tooth as would a low calcium high phosphate diet

Since there is a possibility of fluoride poisoning, perhaps this approach indicated by our studies, ought to be investigated also These findings, in a sense, are the direct outcome of theoretical knowledge of mineralization applied to the caries problem and to me are satisfying for that reason I know that the time is late, and thus will not elaborate further.

Armstrong It is now close enough to five o'clock to allow us to bring the meeting to an end I wish to express my deep appreciation to the guests for their attendance their presentations and their discussions We are especially pleased that Professor Dallemagne has been here We have enjoyed and profited from his presentation and his discussion We wish that it were not so far to Belgium and that he could join us at the future meetings

Follis I should like to express the feeling, I am sure, of the guests and members of the sense of appreciation we have for you Dr Armstrong, in arranging the meeting (Applause)

Armstrong Thank you very much